SUITABILITY OF GENES IN *ARABIDOPSIS* AS ABOVE-GROUND FLUORESCENT REPORTERS TO SCREEN FOR CLUBROOT INFECTION

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By

DRISTY ZAMAN

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ABSTRACT

Clubroot is a soil-borne disease that affects plants in the *Brassicaceae* family caused by the obligate parasite *Plasmodiophora brassicae*. Clubroot infection begins in the roots and leads to gall formation, leading to an overall decrease in plant health. In particular, this can be economically devastating with regards to the *Brassica* species, which includes major commercial crops such as broccoli, cauliflower, and canola. Currently there are no reliable methods for detecting clubroot infection without digging up the plant to inspect its roots, which is inefficient and impractical when dealing with large numbers of individuals. The objective of this research was to identify potential reporter genes which could be used to screen for early clubroot infection in the shoot on a large-scale basis using a fluorescent non-destructive method. 19 genes in *Arabidopsis thaliana*, also belonging to the *Brassicaceae* family, which were either up- or down-regulated during infection according to RNA-Seq data were chosen for testing. A time course consisting of 0, 2, 5, 7, 14, 21, and 28 days post infection was established and gene expression on these days was observed with RT-PCR and RT-qPCR. 8 genes were shown to have coinciding expression trends between the RNA-Seq, RT-PCR, and initial RT-qPCR data, and their promoters were selected to be cloned into reporter vectors. tdTomato and mOrange2 were chosen as fluorescent reporters for their brightness and photostability. A final promoter-reporter construct, *GASA6::mOrange2*, was transformed into *Arabidopsis* and T_1 seeds generated transgenic lines ginger1 and ginger2. Continued RT-qPCR investigation and cloning were conducted concurrently. Unfortunately, final RT-qPCR data revealed that there was no significant difference in expression between control and infected plants for any of the potential reporter genes. The functions of these genes were discussed to evaluate their connection to clubroot disease, and as possible indicators for other potential infection reporter genes. Though inconclusive, results of this research provide insight into the gene expression dynamics in shoot tissue during clubroot infection.

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ABBREVIATIONS

Term	Abbreviation
Abscisic acid	ABA
Aluminum induced protein	AILP1
Analysis of variance	ANOVA
Anthocyanin 5-O-glucosyltransferase	UGT75C1
Ascorbate peroxidase	APX
Auxin Response Factor	ARF
Base pairs	BP
Beta-galactosidase 1	BGAL1
Catalase	CAT
Cauliflower Mosaic Virus	CaMV35S
Cell wall-plasma membrane linker protein	CWLP
Chalcone synthase	CHS
Days Post Inoculation	DPI
Dihydroflavonol 4-reductase	DFRA
Electron transport chain	ETC
Endoplasmic reticulum	ER
Endoxyloglucan transferase	EXGT-A1
Expansin 1	EXP1
Expansin 8	EXP8
Farnesoic acid carboxyl-O-methyltransferase	FAMT
Fluorescent protein	FP
Fragments Per Kilobase per Million	FPKM
GA Insensitive	GAI
Gibberellin	GA
Gibberellin stimulated Arabidopsis 6	GASA6
Glutathione s-transferase 26	GST26
Glycoside hydrolase 3	GH3
Green fluorescent protein	GFP
Guaiacol peroxidase	GPX
Hydrogen peroxide	H_2O_2
Hydroxyl ion	OH•
Indole-3-acetic acid	IAA
Jasmonic acid	JA

Leu-Arg-Asp-Arg	LRDR
Lipid droplet associated protein 1	LDAP1
Mitogen activated protein kinase	MAPK
Nitric oxide	NO•
Nitric oxide synthase-like	NOS-like
Nucleotide diphosphate	NDP
Peroxynitrite	$ONOO^-$
Photosystem I	PSI
Photosystem II	PSII
Potential reporter gene	PRG
Proline-rich proteins	PRP
Pulsed-field gel electrophoresis	PFGE
Qua Quine Starch	QQS
Quantitative trait loci	QTL
Reactive nitrogen species	RNS
Reactive oxygen species	ROS
Red fluorescent protein	RFP
Salicylic acid	SA
Senescence associated and QQS related	SAQR
Sinapoylglucose malate sinapoyltransferase	SNG1
Singlet oxygen	$1O_2$
Small auxin-up RNA	SAUR
Superoxide anion	O_2^-•
Superoxide dismutase	SOD
Tetratricopeptide repeat	TRP
Triticum aluminum 18	TAl-18
Tyr-Gly-Leu	YGL
Xanthine oxidase	XOD

1. INTRODUCTION

1.1 Family *Brassicaceae*

One of the largest eudicot families, *Brassicaceae* is composed of 3709 species and 338 genera (Warwick et al., 2006). Within these, the *Brassica* genus contains many commercially important crops, including broccoli, cauliflower, wild cabbage, and canola. Six species of *Brassica* make up the majority of these crops and their relationship can be elucidated using U's triangle (Nagaharu, 1935). *B. rapa, B. nigra,* and *B. oleracea* are all diploid and make up the three vertices of the triangle. If these species are A, B, and C, respectively, then the hybrids of each of these can be designated AB, BC, and AC. These allotetraploid hybrids, *B. juncea* (AB), *B. carinata* (BC), and *B. napus* (CA), are the other three major crops and fall in the middle of vertices in U's triangle (Cheng et al., 2015). These plants provide edible roots, leaves, buds, flowers, and seeds (Rakow, 2004). *B. carinata* is known as a leaf vegetable and has multiple cultivars with edible leaves. As do cultivars in *B. oleracea,* which also contains edible Brussels sprout buds and the flowering head of broccoli. Seeds provide a variety of uses; for example, they can be ground and used for cooking or processed to make oil. *B. nigra* and *B. juncea* seeds are ground to make spices, and *B. rapa* seeds produce oil. *B. napus* seed is cultivated for the production of canola oil, one of Canada's most agriculturally significant crops. *Brassica* crops have numerous health benefits for humans; they are considered a source of antioxidants and contain metabolites which have been effective in the chemoprevention of cancer (Kapusta-Duch et al., 2012). Investigation into *Brassica* evolution and development can provide invaluable information in regard to maximizing crop production.

1.1.2 *Arabidopsis thaliana*

Arabidopsis thaliana is a flowering plant in the *Brassicaceae* family used widely in research as a model organism. Its relatively short life cycle, high seed number, and small approximately 157 megabase nuclear genome (Bennett et al., 2003) make it an attractive model for genetic, biochemical, developmental, and physiological studies. Its popularity as a model organism was further established through the creation of successful transformation protocols (Meinke et al., 1998).

Several genome databases for *Arabidopsis* available for reference, an example of which is The Arabidopsis Information Resource (Phoenix Bioinformatics TAIR, https://www.arabidopsis.org/index.jsp) which provides not only genomic sequences but compilations of information regarding enzyme pathways, polymorphisms, and microarray data sets. This extensive collection of data facilitates the connection between *Arabidopsis* genomics, proteomics and metabolomics. These data sets can also be used to perform weighted correlation network analysis, such as those used for differential co-expression analysis. These tools are exceptionally useful to study other *Brassicaceae* plants, specifically in the agriculturally relevant *Brassica* family. Comparative genomics have discovered multiple quantitative trait loci (QTL) between *Arabidopsis* and *B. rapa* (Kole et al., 2001), and between *Arabidopsis* and *B. oleracea* corresponding to flowering time (Lan & Paterson, 2000). Additionally, an anther-specific protein is coded by the same gene, *A6,* in both *B. napus* and *Arabidopsis*. These shared loci and corresponding genes suggest that many other *Brassica* plants may also share functional and developmental traits with *Arabidopsis*, making *Arabidopsis* an ideal substitute for the study of *Brassica* species. The use of sequencing tools and these databases can allow us to identify typical changes in gene expression, such as those during different stages of development, as well as changes in gene expression due to external stressors (Borkotoky et al., 2013).

1.2 Stress in plants

Like many organisms, plants must regularly adapt to changes in their environment to maintain homeostasis. When these changes result in the negative impact of metabolism, growth, or development the plant is under stress. Stress can occur through abiotic or biotic means, or a combination of both, and results in a stress response from the plant. Abiotic stresses involve any

change in the environment that originate from non-living factors. For plants these include but are not limited to light, temperature, salinity, heavy metal toxicity, drought, and floods. High radiation or soil contamination are other less common abiotic stressors. Biotic stresses are caused by any living organism. These could take the form of bacteria, fungi, viruses, and parasites. In field settings, it is likely that plants face a combination of biotic and abiotic stressors simultaneously. Caterpillars are an example of both a biotic stress and abiotic stress: a pest that with mechanical wounding (Reymond, 2000). In other cases, one stress can exacerbate another, as seen in heightened viral sensitivity in *N. benthamiana* under cold stress (Szittya et al., 2003). The mechanisms for addressing these changes are extremely important, as plants are largely sessile and cannot relocate to a more suitable environment.

To alert the plant of these changes, the stress response activates once the plant has reached a threshold for macromolecular damage, causing subsequent changes in gene expression. The presence of a threshold allows for fluctuations in the concentrations of molecules involved in regular plant function without triggering a stress response. This initial response to any kind of stress is a short-term solution to minimize cell damage while more long-term stressor-specific genes are activated (Kültz, 2003). Different cell types can have their own damage threshold for specific stresses (Dinneny et al., 2008; Kültz, 2005), contributing to the diversity of stress tolerance between plants.

Several changes occur at the cellular level during stress responses, many of which are involved in stress signaling or signal pathway mediation. One of the major changes involved in almost all stress responses is the change in redox potential in the cell and an increase of reactive oxygen and nitrogen species (ROS and NOS, respectively) as a result of oxidative and nitrosative stress (Adler et al., 1999). Reduction/oxidation reactions, or redox reactions, are necessary for a number of cellular processes in the cell and usually function as a redox pair. Redox pairs have a redox potential which is a measure of their ability to acquire electrons. When this ratio between redox pairs is altered there is an increase in the level of oxidized molecules in the cell. This increase in ROS production may be one of the first signals to indicate the presence of stress and activate the initial stress response (Desikan, 2001). A number of other common acclimation strategies include changes in cell membrane composition (Jang et al., 2004; Welti et al., 2002), an increase of regulatory proteases (Adam, 2001), inhibition of cell proliferation (Skirycz et al., 2011), and induction of programmed cell death or apoptosis (Watanabe & Lam, 2008).

Generally, *Arabidopsis* stress responses can have very specific effects on changes in gene regulation and plant function depending on the underlying cause of the stress. This becomes apparent when you compare abiotic-stressor responses to biotic-stressor responses, though there are genes associated with stress responses which are shared among multiple stressors (Borkotoky et al., 2013). However in a field environment, despite shared responses, a combination of stressors can have an entirely unique effect on the plant distinct from the addition of responses from the same stressors affecting individual plants observed in a lab setting (Mittler, 2006).

1.2.1 Reactive oxygen and nitrogen species

One of the most universal responses to abiotic or biotic stress is the production of reactive oxygen species, or ROS. Molecular oxygen ($^3O_2$) exists as a free radical, with two unpaired electrons that have the same quantum spin. This constrains the ground state triplet molecule to only oxidizing other nonradical atoms or molecules one electron at a time, which leads to highly reactive intermediate products (Halliwell & Gutteridge, 1985). These intermediates can react with cell components and DNA in various ways to damage and disrupt normal cell function.

The superoxide anion (O_2^-•) has accepted a single electron and is the precursor oxidant of most ROS. In plants, the major site of production of O_2^-• is Photosystem I (PS I) in the thylakoid membrane electron transport chain (ETC) located in the chloroplast. All light-dependent reactions required for photosynthesis take place in the thylakoid ETC. Oxygen generated earlier in the transport chain can accept electrons passing through the photosystems and reduce it to O_2^-•. Due to the redox nature of the thylakoid light harvesting complexes, there is always a normal low-level production of O_2^-• in the chloroplast. Under normal conditions, PS I transfers electrons to the protein ferredoxin which are then passed to $NADP^+$ reductase. However, if there are an excess of electrons in the transport chain it has been found that some electron flow is also diverted from ferredoxin to O_2 (Wise & Naylor, 1987), resulting in O_2^-• production due to stress.

The other two major sites of O_2^-• production are in the mitochondria and the peroxisome. Electrons in the mitochondrial ETC have enough energy to directly reduce O_2, with localized reduction at Complex I and III (Turrens, 1997). In the peroxisome matrix of leaves, O_2^-• is generated when xanthine is converted to uric acid by xanthine oxidase (XOD) (Sandalio et al., 1988).

The radical is also generated in the cytosol by the small peroxisomal ETC, which uses O_2 as an electron acceptor (Del Río, 1998).

Both radical and non-radical ROS and NOS can originate from reacting with O_2^-•. Dismutation of O_2^-• to hydrogen peroxide (H_2O_2) is facilitated by the metalloenzyme superoxide dismutase (SOD) in both the chloroplast (Takahashi & Asada, 1988) and the mitochondria (Quan et al., 2008). H_2O_2 can also be produced in peroxisomes during the photorespiration glycolate oxidase reaction, fatty acid β-oxidation, the enzymatic reaction of flavin oxidases, and the disproportionation of superoxide radicals (Corpas et al., 2001). This H_2O_2 can further undergo a Fenton reaction, wherein it reacts with a metal ion (Cu^+ or Fe^{2+}) to produce hydroxyl radicals (OH•) (Fenton, 1894). Additionally, O_2^-• and H_2O_2 can undergo a Haber-Weiss reaction to also form OH• (Phaniendra, Jestadi, & Periyasamy, 2015). Nitric oxide (NO•), a reactive nitrogen species (RNS) formed by nitric oxide synthase-like (NOS-like) proteins, can react with O_2^-• to form peroxynitrite ($ONOO^-$) (Beckman & Koppenol, 1996).

Singlet oxygen ($^1O_2$), unlike other ROS, is produced from an excess of light energy rather than electron transfer. When chlorophyll does not utilize all its absorbed light energy the remaining energy maintains it in a lower state of excitement, known as the chlorophyll triplet state. In the absence of other quenchers to mitigate this excess energy, the chlorophyll triplet reacts with oxygen to create the highly reactive $^1O_2$ (Krieger-Liszkay, 2005). Under normal conditions $^1O_2$ is continuously produced at a low level by Photosystem II (PS II) during photosynthesis (Apel & Hirt, 2004).

Since the presence of these oxidants is inevitable in at least small amounts, many antioxidant strategies exist within the cell to maintain normal levels. Among them, one of the most direct is the dismutation of O_2^-• to H_2O_2 by SOD. This both removes the O_2^-• to prevent OH• formation, and allows for catalase (CAT), ascorbate peroxidase (APX), and guaiacol peroxidase (GPX) to detoxify H_2O_2 to H_2O (Apel & Hirt, 2004). Non-direct methods mitigating ROS production include carotenoids present nearby chlorophyll molecules to act as a quencher for excess photoenergy, in turn preventing $^1O_2$ production (Krieger-Liszkay, 2005). Other radicals like OH• have no dedicated scavenging molecules or preventative mechanisms and high levels of production can result in cell death (Vranová et al., 2002). When stressors effect an increase in ROS/NOS production large

enough to cause a significant imbalance between oxygen species and antioxidant defenses, this is referred to as 'oxidative stress'.

While this increase in ROS/NOS production can be harmful, it can also act as the starting point in signaling pathways to mediate stress. In plants, H_2O_2 is known to activate mitogen activated protein kinase (MAPK) signaling components (Jonak et al., 2002). Overexpression of H_2O_2 mediated MAPKKK ANP1 in transgenic *Arabidopsis* was shown to exhibit better tolerance heat shock, freezing, and stress than the wildtype (Kovtun et al., 2000). Overexpression of nucleotide diphosphate (NDP) kinase 2, another kinase in the MAPK signaling pathway, also exhibited increased tolerance to cold, salt, and oxidative stress (Moon et al., 2003). Through DNA microarrays and cDNA profiling, 175 *Arabidopsis* genes were found to change expression under oxidative stress—of these, 113 induced genes coded for antioxidant proteins or other stress defense responses (Desikan, 2001).

1.2.2 Plant hormones

Plant hormones, or phytohormones, are organic compounds synthesized endogenously and dispersed within the organism. Involved in all levels of cellular organization, rapid changes in hormone homeostasis can signal a stress response. Although signal transduction is not clear in some pathways, it is generally acknowledged that physiological hormone responses are regulated by phosphorylation and phosphatases of proteins through kinases (Hirt, 1997; Raz & Fluhr, 1993). Crosstalk between a variety of hormones including auxin, cytokinin, gibberellin (GA), abscisic acid (ABA), salicylic acid (SA), ethylene, and jasmonic acid (JA) are essential for plants to regulate cell development.

Auxin is known to induce gene expression in three gene families for the regulation of cell growth: Aux/IAA family, GH3 (glycoside hydrolase 3) family, and small auxin-up RNA (SAUR) family (Woodward & Bartel, 2005). Indole-3-acetic acid (IAA), a major auxin involved in all major aspects of plant growth and development, is highly regulated through multiple pathways in the cell (Strader & Bartel, 2008; Woodward & Bartel, 2005). Auxin induced Aux/IAA proteins bind to auxin producing Auxin Response Factors (ARF) to inhibit auxin transcription through negative regulation (Mockaitis & Estelle, 2008; Szemenyei et al., 2008). Pathogen induced upregulation of auxin activates

cell wall modification proteins, generally expansins, to loosen the cell wall (Ding et al., 2008). The loosening of the cell wall facilitates easier access for disease causing agents such as *Agrobacterium tumefaciens* (Yamada, 1993). Conversely, blocking auxin expression has shown to increase pathogen resistance (Wang et al., 2007).

Cytokinin is involved in vascular differentiation, seed development, leaf senescence, stress tolerance, and many other plant functions (Müller & Sheen, 2007). Generally, cytokinin is downregulated under stress conditions. This is seen in plants exposed to water limiting conditions (Argueso et al., 2009) and pathogen infection (Siemens et al., 2006). A possible method of signal transduction could involve a decrease in shoot cytokinin concentration that results in changes in shoot gene expression (Hare et al., 1997). It has been found that glucoside-cleaving β-glucosidase is produced by many biotrophic and hemibiotrophic fungal pathogens (but not necrotrophic pathogens) to cleave zeatin-*O*-glucoside and release active zeatin (Cooper & Ashby, 1998). This accumulation of cytokinin at the site of infection is likely to direct host nutrients to the pathogen (Cooper & Ashby, 1998; Murphy et al., 1997). Cytokinin and another relevant phytohormone, ABA, have an intensely antagonistic relationship and a change in cytokinin-related gene expression is likely to reflect in the modulation of ABA (Ha et al., 2012).

Among other functions, GAs regulate seed development, organ elongation, and flowering time (Yamaguchi & Kamiya, 2000). Similar to auxin, GA induces genes which downregulate its own production (Fleet & Sun, 2005). DELLA proteins, negative regulators of growth, are degraded via GA production (Bari & Jones, 2009). DELLA proteins are involved in the mediation of pathogen induced stress response through pathways involving SA, JA, and ethylene (Achard et al., 2006). They have also been shown to modulate ROS signaling in the presence of both biotic and abiotic stress (Achard et al, 2008).

ABA, SA, JA, and ethylene are known to regulate plant responses to both biotic and abiotic stress. ABA in particular, has one of the fastest responses to abiotic stress (Nakashima et al., 2009), resulting in stomatal closure. ABA also plays a role in cell wall modification (Flors et al., 2008) and the induction of catalase (Xing et al., 2008), both of which are responses to abiotic and biotic stressors. The increase of SA levels were seen to enhance pathogen tolerance, and conversely the decrease of JA was seen to impede pathogen tolerance (Brodersen et al., 2006; Petersen et al., 2000).

JA and ethylene are known to work synergistically, with a number of stress defense genes upregulated by both hormones (Schenk et al., 2000). It is the complex synergistic and antagonistic crosstalk that occurs between these hormone pathways that allows for the mediation of an incredible number of both biotic and abiotic stresses.

1.3 Clubroot

A major biotic stress for *Brassicaceae* plants that is prevalent in over 60 countries around the world is clubroot. It is a soil-borne disease that affects plants in the *Brassicaceae* family, which includes major commercial crops in the *Brassica oleracea* species (broccoli, cauliflower, etc.) and *Brassica napus* (canola). It is caused by the obligate parasite *Plasmodiophora brassicae* Woronin, which is classified as a protist in subgroup Rhizaria (Burki et al., 2010). Since its identification in Canada in 1997 (Morasse et al., 1997), the presence of *Plasmodiophora* in crop fields has steadily increased (Strelkov et al., 2011) and has a devastating effect on crop yield and health.

The clubroot pathogen has two distinct stages: infection of the root hairs (primary phase), and infection of the cortical root and gall formation (secondary phase) (Cook & Schwartz, 1930). A third preliminary phase can be considered spanning its lifecycle in the soil from the resting spores to the discharge of zoospores (Ayers, 1944). Infestation begins when clubroot infected tissue is broken down by bacteria and fungi, releasing sporangia which are then dispersed through the soil, usually through rain. These spores are around 3 μm large, roughly spherical in shape (Buczacki & Cadd, 1976), and have spines (Sharon & Icpjabola, 1967). Sporogenesis involves the breakdown of the multinucleate vegetative plasmodium, the final stage of the pathogen, into multiple uninucleate sporangia. The mature resting sporangium is rich in lipids, and contains the organelles in the vegetative plasmodium as well as some organelles from the host cell (Sharon & Icpjabola, 1967).

The germination of spores can be initiated in 1-10 days by leaving spores in tap water (Ayers, 1944), but have been shown to germinate with more success with the introduction of *Brassica* exudates (Macfarlane, 1970), which is the usual germination signal for *Plasmodiophora*. Once germinated, two flagella, one long and one short, emerge from an invagination in the plasma membrane of the sporangia. These flagella are followed by the uninucleate protoplast cell body of a motile spore, or a primary zoospore (Tanaka, et al., 2001). Once the zoospore reaches the root hairs

there is a two hour period wherein the flagella and axonemes are retracted, and a Stachel forms in the cavity. The cavity, or Rohr, is then rapidly evaginated, thrusting the Stachel outwards, puncturing the cell wall and inserting the zoospore into the host—all occurring within one minute (Aist & Williams, 1971).

The zoospore within the root hair host cell is amoeba-like and moves slowly to a cell suitable for further development. Once the zoospore has stopped within a cell and starts enlarging, its nucleus starts dividing via protomitosis (Cook & Schwartz, 1930). This primitive form of mitosis exhibits a cruciform shape made by chromatic aligned perpendicular to the nucleolus, and as such is known as "Cruciform division" (Dylewski & Miller, 1984). At this stage, multiple amoeba can aggregate together to create a larger primary plasmodium, and protomitosis continues in daughter nuclei (Cook & Schwartz, 1930). From 3-8 days after infection, mature primary plasmodium cleaves into zoosporangia, which contain 4-16 secondary zoospores each (Hwang et al., 2012). These secondary zoospores are then expelled out of the host cell through an opening made by the zoosporangium attached to the cell wall (Ayers, 1944). This concludes the primary phase of infection.

The secondary stage begins with the infection of secondary zoospores in the cortical tissues of the roots. So far, it is unclear whether the secondary zoospores travel through the soil to the cortical roots or if they move through the root hair, or a combination of both. Once penetration has occurred, secondary plasmodia enlarge, leading to cellular hypertrophy, hyperplasia, and gall formation. This aggregation of plasmodia create tumors, or "clubs" in the roots, giving clubroot its name. The many small binucleate secondary plasmodia in the cortical cells undergo synchronous mitotic divisions to become multinucleate, with fusion of haploid nuclei to make diploid nuclei (Ingram & Tommerup, 1972). Meiosis follows this fusion and subsequently the mature plasmodia are cleaved to make new sporangia (Kageyama & Asano, 2009).

1.3.1 Physiological responses

By far the most notable symptom of clubroot is spindle shaped gall formation, caused by the accumulation of plasmodia infected cells at the infection site. Not only the increase in number of cells, but the larger size of both infected cells and cells nearby the infection site contribute to the

formation of clubs. By day 13 of infection, plasmodia have penetrated the root cambium and both parasite and host cells have accelerated nuclear and cell division, as well as a significant increase in growth compared to normal cells (Kunkel, 1918). Once the plasmodia are in the cambium layer, young cells offer little resistance and the amoeba infects the cambium cells easily. Additionally, the ingression of plasmodia into cambium cells compromise the growth of vascular elements, resulting in a conducting system which cannot contend with the leaf surface area (Kunkel, 1918). The two mechanisms of infection are highlighted in these methods: direct infection through plasmodium movement through the cambium layer, and indirect infection through host cell division (Kunkel, 1918). It is the formation of these galls that divert nutrients and water uptake that result in the stress symptoms seen in infected plants (Dixon, 2006).

During infection, a number of genes are differentially expressed in both shoots and roots of the affected host. Changes in expression in over a 1000 *Arabidopsis* genes are present as early as 10 days post inoculation with clubroot spores, before any symptoms are even visible (Siemens et al., 2006). Among them, several plant hormone pathways are influenced by clubroot infection. Cytokinin, well known for promoting cell division, is initially more active in clubroot tissues (Dekhuijzen & Overeem, 1971) and present in significantly higher concentrations in clubroot infected roots (Dekhuijzen, 1981) as seen in *B. campestris*. The occurrence of different forms of cytokinins in pathogen and host and their allocation also suggests that the plasmodium are exporting cytokinin into the host and directly affecting cytokinin concentration (Dekhuijzen, 1981). Pathogen cytokinin transfer may account for the downregulation of host cytokinin homeostasis genes (Siemens et al., 2006).

Auxins, another plant hormone well known for regulating plant growth, were not found to be produced in the pathogen. However host regulation of auxin pathways was observed to be dependent on the stages of pathogen infection (Ludwig-Müller, 1999). During initial infection and stimulated root growth, auxin production increased to allow for cell expansion (Devos et al., 2005). Late in the infection stage, during gall development, concentration of auxin in *B. rapa* roots decreased and concentration of auxin hydrolases increased (Schuller & Ludwig-Müller, 2006). The reduction of cytokinins and auxins in the late stages of infection are likely due to the plant hormone sink established by the gall.

Jasmonic acid has been found to regulate plant response to both biotic and abiotic stressors (Wasternack, 2007). Specifically, JA increases the production of indole glucosinolates (Bodnaryk, 1994), which are known to induce signaling pathways in response to tissue damage that result in the upregulation of detoxifying enzymes (Agerbirk et al., 2009). JA in clubroot infection was seen to be highly upregulated in both shoots and roots, however glucosinolate biosynthesis is upregulated in shoots and downregulated in roots in the late infection stage (Irani et al., 2018). This may indicate failing pathogen defense in the root, whereas the shoot experiences infection symptoms later and employs delayed stress responses compared to the root.

Proteomic studies have revealed changes in protein abundance in both *Arabidopsis* and canola between clubroot infected and control roots. Proteins involved in cell defense, cell differentiation, and detoxification of ROS were altered in *Arabidopsis* (Devos et al., 2006). Proteins with roles in lignin biosynthesis, cytokinin metabolism, glycolysis, intracellular calcium homeostasis, and ROS detoxification were among the 20 proteins altered in canola (Cao et al., 2008).

Sucrose and starch pathways have been associated with *Plasmodiophora* infection in *Arabidopsis* (Brodmann et al., 2002). A high concentration of starch grains are found in *Plasmodiophora* infected cells in the early stages of infection, which are largely absent in mature plasmodia (Cook & Schwartz, 1930). However there is an upregulation in many starch biosynthesis pathways in late infection stages in root tissue (Irani et al., 2018), which may be a defense to counteract the root gall acting as a nutrient sink. *B. oleracea* hypocotyl tissue in infected plants contained a much higher concentration of glucose, fructose, mannose, and trehalose than in healthy hypocotyls, but interestingly galactose concentrations displayed the opposite trend (Keen & Williams, 1969).

Analogous with the major changes in cell size, several changes in gene expression occur in cell wall genes. Many cell wall component genes and cell wall degradation proteins were downregulated in shoot tissues (Irani et al., 2018). Conversely, there was an upregulation of cell modification genes in roots, many of which are part of the cell wall loosening alpha-expansin gene superfamily (Sampedro & Cosgrove, 2005). The downregulation of cell wall degradation enzyme genes in roots suggests that as an obligate parasite, *Plasmodiophora* prioritizes infection proliferation over cell degradation.

The final stages of disease are observed best in the aboveground tissue, as gall formation puts the shoot in a highly stressed state. Early stages of infection present as dwarfing, with overall shorter roots and smaller shoots (Cook & Schwartz, 1930), however once the gall is formed, the effects of nutrient deficiency and drought can overwhelm the host. The primary macronutrients required for healthy plant growth, nitrogen, potassium, and phosphorous are essential. Among their many functions they are integral to amino acid and chlorophyll synthesis (Olday, 1972), plant cellular homeostasis (Maathuis & Sanders, 1996), and energy conservation and metabolic regulation (Raghothama & Karthikeyan, 2005). An increase in H_2O_2 production, a stress response, was shown to correlate with deficiencies in any one of these nutrients (Shin and Schachtman, 2004; Ryoung et al., 2005). Drought responses will result in the production of abscisic acid (ABA), which causes stomatal closure and induces other stress response genes. Other responses include cell growth and photosynthesis arrest, and activation of cellular respiration (Shinozaki et al., 2003). Ultimately, due to the loss of vascular tissue, the host will wilt and die from lack of sufficient water and the nutrients it delivers.

1.3.2 Genomics

Historically the *Plasmodiophora* genome has been difficult to study, as inability to produce the pathogen axenically and small chromosomes (Siemens et al., 2009) . Electron microscopy and pulsed-field gel electrophoresis (PFGE) (Ito et al., 1994) estimated the genome size to be 18-20.3 Mb. Recent genome sequencing has revealed the pathogen genome to be approximately 24 Mb (Schwelm et al., 2015). Techniques such as suppression subtractive hybridization have allowed for the isolation of specific *Plasmodiophora* genes, which may provide insight into genes that cause clubroot disease (Bulman et al., 2006). Unfortunately, multiple pathotypes make it difficult to draw conclusions between genetic makeup and virulence patterns. Thus far, transcriptome studies have generally been focused on the host response to clubroot (Jia et al., 2017; X. Zhang et al., 2016) and have highlighted the changes in gene expression as early as 24 hours into infection (Zhao et al., 2017).

1.3.3 Impact in agriculture and current research

Canola production and processing is one of the most important and lucrative agribusinesses in Canada. There are numerous direct and indirect economic benefits that stem from the canola industry. Canola seed crushing, over 50% processed domestically, netted approximately C$430 million between 2007-2008 and 2009-2010. Oil refining adds value to crude canola oil, grain and product handling add value to canola seed, and unprocessed canola seed is exported (Rempel et al., 2014). The indirect value through providing employment is also a great benefit to the Canadian economy.

The first outbreak of clubroot in Canada canola was in Quebec in 1997 (Pageau et al., 2006) and it resulted in upwards of 80% grain loss yields. Western Canada saw its first outbreak in Alberta in 2003 (Tewari et al., 2005), and infestations in Canadian fields have risen steadily since then. In 2012 alone, 233 total fields in Alberta were discovered to be clubroot infested (Strelkov et al., 2012). Clubroot infestations have even been dispersed to neighbouring provinces Saskatchewan (Dokken-Bouchard et al., 2008) and Manitoba (Rempel et al., 2014). It is presumed that the transfer of spores is via field equipment (Strelkov et al., 2011). Though the loss of horticultural crops are hard to quantify, an estimated yield loss of 30-100% have occurred in severely infected crops in Alberta (Strelkov et al., 2007). These losses can also occur from secondary effects, such as delayed maturity resulting in no market value or unattractive appearance and size resulting in lowered market value (Dixon, 2009). Canada is not the only country with investments in canola production. All Chinese cabbage cultivars are at risk of infection, and significant losses have been seen in plant height and seed production in infected crops (Jing et al., 2008). In Australia up to 50% loss of yield of oil seed can be contributed to clubroot infection (Donald & Porter, 2003). Additionally, resting spores can survive in the soil for up to 20 years (Wallenhammar, 1996) and render a plot of farmable land unusable for *Brassica* crops.

With the potential for a significant negative impact in the economy if canola industry is put in jeopardy due to clubroot, there are endeavours being made to cultivate resistant *Brassica* strains. Unfortunately, resistance to *Plasmodiophora* is pathotype specific, and there are no *Brassica* species resistant to every pathotype (Hwang et al., 2012). Identifying clubroot infected plants is one of the major challenges of investigating *Plasmodiophora* infection. The spores are difficult to detect due to

their small size, and the pathogen cannot be cultured. In lab conditions the model organism *Arabidopsis* has been a useful host to study infection. *Arabidopsis* and *Brassica* are both in the *Brassicaceae* family so their level of synteny results in similar genes responses to clubroot infection. A *B. rapa* linkage map with 10 linkage groups was compared to *Arabidopsis* and both plants were found to contain major clubroot resistance genes (Suwabe et al., 2006).

In field situations, infection is usually not apparent until the infection is already underway, and signs of dwarfing are present. Confirmation of infection is achieved by uprooting the host to expose the clubbed roots, which is an ineffective and costly method (Faggian & Strelkov, 2009). Microscopic examination (MacFarlane, 1952) and spore staining (K. Takahashi & Yamaguchi, 1988) have been shown to work, however they are time consuming and require training to conduct. PCR (Cao et al., 2007) and qPCR (Sundelin et al., 2010) based tests are efficient alternatives to identifying pathogen presence, and PR (pathogenesis-related) genes can be used to detect infection in the host, as they have been shown to be differentially expressed during clubroot infection in both shoots and roots (Lovelock et al., 2012), but to date there is no effective method for identifying infection or surveying disease progression specifically in the shoots.

1.4 Reporter genes

A highly efficient method of investigating gene expression is through the use of reporter gene constructs. These consist of genes whose protein products cause a visible change, but which do not alter normal cell function or cause changes in morphology, fused downstream of a DNA coding sequence, making them easy to identify. This attribute can also be used to confirm successful transformations. A variety of reporter genes exist, including GUS assays, blue-white screens, and fluorescent proteins. GUS reporter gene assays are based on β-glucuronidase, which will fluoresce when stained with certain dyes. GUS reporter gene assays also commonly use X-gluc (Jefferson et al., 1987) as a dye. These assays are useful because they do not damage tissue (Gould & Smith, 1989). A simple example of a reporter gene is the blue-white screens in bacterial cloning experiments, which are based on the expression of β-galactosidase and are used to identify successfully transformed cell colonies. Transformed organisms are plated on X-gal, which cause β-galactosidase expressing cells to turn white through the lactose operon pathway (Macgregor et al., 1991).

The benefit of using of fluorescent protein (FP) reporter constructs is that they can be observed in live tissue. Promoter-reporter constructs can illustrate the degree of gene of interest expression in real-time, which allows for the observations of differentially expressed genes during normal development and stress situations. Similarly, protein-reporter constructs can be used to investigate protein localization (Chalfie et al., 1994). Essential characteristics of an effective fluorescent protein include: a bright signal with minimal autofluorescence, sufficient photostability, no oligomerization with genes or proteins of interest, insensitivity to external stimuli, no toxicity to organism of interest, and no crosstalk between FPs when using multiple reporters (Shaner et al., 2005). The green fluorescent protein (GFP) discovered in jellyfish *Aeuorea victoria* (Tsien, 1998) has been a widely used reporter due to its brightness and stable half-life (Suto & Ignar, 1997). Additionally, the production of GFP in the cell has no cytotoxic effects or fitness costs (Harper et al., 1999). Many GFP derivatives have been produced (van Roessel & Brand, 2002), and a GFP red fluorescent protein (RFP) orthologue DsRed purified from *Dicosoma* (Matz et al., 1999). A non-GFP variant FP is flavin-based iLov, Though the full understanding of their utility is unknown, the advantages of iLov are its smaller size compared to GFP and its reversible photobleaching (Chapman et al., 2008). There is ongoing enhancement of FPs to maximize their effectiveness in imaging live cells and tissues (Chudakov et al., 2010; Cormack et al., 1996; Shaner et al., 2004).

1.5 Objectives

The goal of my research is to identify possible genes which will act as an indicator of early clubroot infection in above-ground tissue using a non-destructive fluorescence-based method. In accomplishing this, my objectives are as follows:

1. Select potential gene reporters (PRGs) based on available RNA-Seq data (Irani et al., 2018)
2. Establish a time course for these genes during clubroot infection
3. Evaluate suitability of PRGs for detection of clubroot infection with RT-PCR and RT-qPCR
4. Construct promoter-reporter vectors using one or more PRG promoters
5. Transform *Arabidopsis* with vector constructs via *Agrobacterium*-mediated transformation;

In the longer-term, these transformed lines of *Arabidopsis* may provide a valuable tool in mutant screens to investigate genes involved in clubroot infection, and ultimately aid in the development of clubroot resistant *Brassica* crops.

2. MATERIALS AND METHODS

2.1 Plant material and growth conditions

A time course was used to identify changes in gene expression after inoculation of *Arabidopsis thaliana* with clubroot spores. Wildtype Col-0 *Arabidopsis* seeds were incubated at 4°C for four days prior to planting. Seeds were planted in soil and grown on a long-day cycle (16 hours 122 μM of photons m^{-2} s^{-1}; 8 hours 0 μM of photons m^{-2} s^{-1}) at a relative humidity of 65% and 22°C for 10 days. On day 10, seedlings were repotted 4 seedlings to a pot with fresh soil and returned to growth chamber for 4 days to allow for any stress response resulting from the repotting to alleviate. After 4 days some of the plants were inoculated with clubroot resting spores and plant tissue was collected 0, 2, 5, 7, 14, 21, and 28 Days Post Inoculation (DPI). Plant material was either used immediately, or frozen in liquid nitrogen and stored at -80 ºC until used.

2.1.1 Inoculation

Mature galls were collected from canola (*Brassica napus*) infected with clubroot resting spores. The galls were used either dried or fresh depending on availability. Generally, fewer resting spores were collected from dried galls. Approximately 2 g of gall tissue was submerged in 0.25% Tween solutions for 5-7 minutes for cleaning. Tissue was then washed with 70% ethanol and rinsed twice with sterile dH_2O. Samples were ground with a 10% sucrose solution and strained through 8 layers of cheesecloth. The approximate concentration of spores was calculated using a haemocytometer. Each plant was inoculated with 1 mL of sucrose solution containing approximately $4x10^7$ spores. The solution was pipetted gently onto the base of the plant and plants were returned to the growth chamber.

2.1.2 Tissue collection

Two hours after inoculation, three random control pots and three random treatment pots were selected and harvested for 0 DPI samples. Forceps were used to loosen seedlings and seedlings were gently rinsed with water. The shoots and roots were cut and separated into individual 1.5 mL Eppendorf tubes. The tubes were frozen in liquid nitrogen and stored at -80°C for later RNA extraction. The same procedure was followed on days 2, 5, 7, 14, 21, and 28 DPI, however 14, 21, and 28 DPI samples only required harvesting of two pots per control and two pots per infection due to the older plants providing more tissue. The entire shoot was collected on 0, 2, 5, and 7 DPI. Sections of old and new leaf tissue from each plant were collected for 14, 21, and 28 DPI.

2.2 RNA-Sequencing

Irani et al. (2018) collected clubroot inoculated shoots and roots at DPI 17, 20, and 24 and generated an RNA-Sequencing (RNA-Seq) library. RNA was extracted using a TruSeq RNA sample preparation kit (Illumina, California, USA). RNA sequencing was carried out in inoculated and control plants with five plants per replicate, with three biological replicates in total. Library sequencing (100 cycles) was conducted from both ends on an Illumina HiSeq 2500 (Illumina). The RNA-Seq data was trimmed using Trimmomatic ver.0.30 (Bolger et al., 2014), aligned to the genome of *Arabidopsis* with Clufflinks and normalized with Cuffdiff (Trapnell et al., 2013).

2.3 Selection of potential reporter genes

Potential reporter genes (PRG) were selected from the shoot RNA-Seq dataset based on parameters which were imposed to select transcripts that had the qualities of a good reporter gene, such as consistent and reliable expression. Changes in gene expression were focused on DPI 20 and DPI 24. Control DPI 20 and control DPI 24 are within 200 FPMK units of each other. Inoculated DPI 20 and inoculated DPI 24 are within 500 FPMK units of each other. Between both DPI 20 (control and inoculated) and DPI 24 (control and inoculated) there is a difference greater than 100 FPMK units. Expression between control and inoculated in both DPI 20 and DPI 24 are different by at least a factor of two.

2.4 cDNA synthesis

RNA was extracted from frozen shoot samples and used to synthesize cDNA for use in gene expression analysis using RT-PCR and RT-qPCR. Frozen shoot tissue from control and inoculated samples were ground using liquid nitrogen. RNA was extracted using the EZNA Plant RNA Kit (Omega Bio-tek, Georgia, USA) according to manufacturer's protocols. Concentration and quality of the RNA was verified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA). All extracted RNA was then diluted to the same concentration for use in cDNA synthesis. RNA not used for cDNA synthesis was stored at -80°C.

cDNA was generated using the QuantiTect Reverse Transcription Kit (Qiagen, Toronto, Ontario, CA) from RNA extracted from control and inoculated samples. Approximately 300 ng of RNA was used for each reaction. The cDNA was diluted 5-fold and stored at -20°C.

2.5 RT-PCR and RT-qPCR primers and protocol

The coding sequence of all Arabidopsis genes was obtained from the plant genome sequence database Phytozome v12.1 (Phytozome version 12.1, https://phytozome.jgi.doe.gov/pz/portal.html). Primers were designed using Primer3 v0.4.0 (Primer3 Input version 0.4.0, http://bioinfo.ut.ee/primer3-0.4.0/). All settings were set to default, except product size, which was set to 100-200 base pairs. All primers generated (Table 2.1) had a T_m within the range of 58-63°C. Various PCR protocols were tested to determine the optimal annealing temperature for all primers. Primers were generated for optimization in RT-qPCR but were also used in RT-PCR. Custom primers were constructed by Invitrogen custom primers (Thermo Fisher Scientific). 100 mM primer stock solutions were diluted to 10 mM or 20 mM working solutions, which were stored at -20°C. Stock solutions were stored at -80°C.

Table 2.1 RT-PCR and RT-qPCR primers. Forward and reverse primers for coding sequence of selected genes are shown 5' to 3'.

Gene ID	Forward Primer	Reverse Primer
At1g27020	GTAACTCAAATCGTCCCGGC	GTGAAACGGGTGCTTTCCAT
At1g64360	AAATGGGCCGAAACATGACC	ATAAACCGGTTCGTCCCAGT
At1g67360	AGCAAGGAGGTAGCGCTAAA	GCTCCTTCGACCTTTTCCAC
At1g69530	TACCCTTGGAGCAATGACGT	TTCCGTATCCACAAGCACCT
At1g74670	TGGAGGACAATGCACAAGGA	ACTTGTTTGTTGCCGTACGT
At1g80130	GTCAAGATGTCCCCGGAAGA	ACATGGTAGTTGGAGGCACA
At2g06850	GTTGCCAAGCTTCTGTGGAA	CCATTTGAGACGACGCCATT
At2g22990	AGTAAAGGAAAATGGGCGCG	AGTTGCAAGAAAAGGGACCG
At2g40610	GGACAAAACTGGCAGAGCAA	GCCAATTAGAAGGAGCCACG
At3g13750	CCCATCACTCCTATGACGCT	TAAGCACAAACGGCTTCCAC
At3g15450	AGAGCTTTGACCATCCGACT	CGCCCAGTTAGCTTCACTTC
At3g22120	AACCTCCCATCGTAAAGCCA	TGTTGGCGGTGTTACAACTG
At3g44860	ATTCACAATGATCGGCGGTG	GTTTGTACCGCGGTGAAAGT
At4g14090	GGTGGGATGGTGTTCTCAGA	CGCCGTTGTACACTGATCAG
At5g09810	GATATTCAGCCACTTGTCTGTGAC	CATGTTCGATTGGATACTTCAGAG
At5g13930	CACGTGTTGAGCGAGTATGG	CACTCCAACCCTTCTCCTGT
At5g17220	GGCTTTCTTCGAACCGGTTT	AACTACCCCGAGCCTTAACC
At5g20190	GCTGCTCCTGAGGACTGTTA	CATGGCCGTGATTGGAGAAG
At5g24490	AGACCATGGAAGGCACATGA	CAGTCAATGGTGGCATCTCG
At5g42800	GACGTCAAAACCGGAGATGG	TCTGTTGTGCTAGCATGGGA

Each solution for the RT-PCR mix contained 2 μL of 10x PCR Buffer, 2 μL of 2 mM dNTP mix, 2 μL of 10x CoralLoad, 0.5 μL of 20 mM forward primer, 0.5 μL of 20 mM reverse primer, 10.9 μL of sterile dH_2O, 0.1 μL of TopTaq DNA polymerase (Qiagen), and 2 μL of cDNA template. The mix was vortexed and spun down, and a MasterCycler gradient thermal cycler (Eppendorf, Hamburg, Germany) was used for PCR amplification. All positive controls for RT-PCR and RT-qPCR throughout the experiment were actin7 (At5g098101). The RT-PCR protocol for amplification of genes was 94°C for 3 minutes for initial denaturation, 30 cycles of 94°C for 30 seconds, 62°C for 30 seconds, 72°C for 30 seconds, for denaturation, annealing, and elongating, then 72° for 10 minutes for a final elongation. PCR products were separated at 110 volts on a 2% agarose gel because of their smaller product size. A 1kb+ DNA ladder was used to confirm presence of desired PCR products based on size (Thermo Fisher Scientific).

Each solution for the RT-qPCR mix contained 2.5 μL of 10x PCR Buffer, 2.5 μL of 2 mM dNTP mix, 1.25 μL of 25 mM $MgCl_2$, 1.25 μL of 20x EvaGreen (Biotum, Scarborough, Ontario, CA), 0.5 μL of 10 mM forward primer, 0.5 μL of 10 mM reverse primer, 0.25 μL of TopTaq DNA polymerase (Qiagen), 14.25 μL of sterile dH_2O, and 2 μL of cDNA template. Samples were loaded into a 96-well tray and gently tapped to mix, and a Bio-Rad iQ 5 thermal cycler (Bio-Rad Laboratories, Mississauga, Ontario, CA) was used for real time PCR amplification. The RT-qPCR protocol for amplification of genes was 95°C for 3 minutes, 45 cycles of 95°C for 20 seconds, 62°C for 30 seconds, 72°C for 30 seconds, then 65°C for 2 minutes and 91 cycles at 50°C for 10 seconds.

2.5.1 RT-qPCR data analysis

The ΔΔCt method (Livak & Schmittgen, 2001) was used to analyze RT-qPCR data. The average Ct value of the housekeeping gene (actin7) in the inoculated samples was subtracted from the average Ct value for the experimental gene in inoculated samples to obtain the ΔCt in inoculated samples. The average Ct value for the housekeeping gene in control samples was subtracted from the average Ct value of the experimental gene in the control samples to obtain the ΔCt in control samples. The ΔCt of control samples was subtracted from the ΔCt of inoculated samples to obtain the ΔΔCt.

2.6 Construction of fluorescent protein reporters

Endoplasmic reticulum (ER) localized tdTomato and mOrange2 fluorescent protein sequences (Figure 2.1) were created using the fluorescent protein sequences found on SnapGene (SnapGene, http://www.snapgene.com/resources/plasmid_files). The tetrapeptide HDEL sequence was added to the C-terminal of the protein sequence for ER-localization. Restriction enzyme cut sites for HindIII and Bsu36I were added, on the N-terminal and C-terminal respectively, to facilitate removal from the gene entry vector and ligation into the destination vector. A 6 base pair CA nucleotide repeat was added to the ends of the sequences as a buffer to avoid digestion of the desired PCR product.

tdTomato and mOrange2 sequences were ordered using Invitrogen's GeneArt service (Thermo Fisher Scientific). Both genes were acquired within plasmid backbones (Figure 2.2) and purified from transformed bacteria. Sequences were 100% congruent at the insertion site. The synthesized tdTomato gene is 1468 base pairs long and inserted in a pMK-RQ-Bs vector backbone. The synthesized mOrange2 gene is 748 base pairs long and inserted in a pMA-T vector backbone.

HindIII restriction enzyme cut site | Bsu36I restriction enzyme cut site | C-terminus HDEL sequence | Fluorescent protein sequence | Nucleotide repeat

tdTomato, 1431 bp

CACACAAAGCTTATGGTGAGCAAGGGCGAGGAGGTCATCAAAGAGTTCATGCGCTTCAAGGTGCGCATGGAGGGCTCCAT
GAACGGCCACGAGTTCGAGATCGAGGGCGAGGGCGAGGGCCGCCCCTACGAGGGCACCCAGACCGCCAAGCTGAAGGT
GACCAAGGGCGGCCCCCTGCCCTTCGCCTGGGACATCCTGTCCCCCCAGTTCATGTACGGCTCCAAGGCGTACGTGAAGCA
CCCCGCCGACATCCCCGATTACAAGAAGCTGTCCTTCCCCGAGGGCTTCAAGTGGGAGCGCGTGATGAACTTCGAGGACGG
CGGTCTGGTGACCGTGACCCAGGACTCCTCCCTGCAGGACGGCACGCTGATCTACAAGGTGAAGATGCGCGGCACCAACTT
CCCCCCCGACGGCCCCGTAATGCAGAAGAAGACCATGGGCTGGGAGGCCTCCACCGAGCGCCTGTACCCCCGCGACGGCG
TGCTGAAGGGCGAGATCCACCAGGCCCTGAAGCTGAAGGACGGCGGCCACTACCTGGTGGAGTTCAAGACCATCTACATG
GCCAAGAAGCCCGTGCAACTGCCCGGCTACTACTACGTGGACACCAAGCTGGACATCACCTCCCACAACGAGGACTACACC
ATCGTGGAACAGTACGAGCGCTCCGAGGGCCGCCACCACCTGTTCCTGGGGCATGGCACCGGCAGCACCGGCAGCGGCAG
CTCCGGCACCGCCTCCTCCGAGGACAACAACATGGCCGTCATCAAAGAGTTCATGCGCTTCAAGGTGCGCATGGAGGGCTC
CATGAACGGCCACGAGTTCGAGATCGAGGGCGAGGGCGAGGGCCGCCCCTACGAGGGCACCCAGACCGCCAAGCTGAAG
GTGACCAAGGGCGGCCCCCTGCCCTTCGCCTGGGACATCCTGTCCCCCCAGTTCATGTACGGCTCCAAGGCGTACGTGAAG
CACCCCGCCGACATCCCCGATTACAAGAAGCTGTCCTTCCCCGAGGGCTTCAAGTGGGAGCGCGTGATGAACTTCGAGGAC
GGCGGTCTGGTGACCGTGACCCAGGACTCCTCCCTGCAGGACGGCACGCTGATCTACAAGGTGAAGATGCGCGGCACCAA
CTTCCCCCCCGACGGCCCCGTAATGCAGAAGAAGACCATGGGCTGGGAGGCCTCCACCGAGCGCCTGTACCCCCGCGACG
GCGTGCTGAAGGGCGAGATCCACCAGGCCCTGAAGCTGAAGGACGGCGGCCACTACCTGGTGGAGTTCAAGACCATCTAC
ATGGCCAAGAAGCCCGTGCAACTGCCCGGCTACTACTACGTGGACACCAAGCTGGACATCACCTCCCACAACGAGGACTAC
ACCATCGTGGAACAGTACGAGCGCTCCGAGGGCCGCCACCACCTGTTCCTGTACGGCATGGACGAGCTGTACAAGCACGA
CGAGCTGTAACCTCAGGCACACA

mOrange2, 711 bp

CACACAAAGCTTATGGTGAGCAAGGGCGAGGAGAATAACATGGCCATCATCAAGGAGTTCATGCGCTTCAAGGTGCGCAT
GGAGGGCTCCGTGAACGGCCACGAGTTCGAGATCGAGGGCGAGGGCGAGGGCCGCCCCTACGAGGGCTTTCAGACCGCT
AAGCTGAAGGTGACCAAGGGTGGCCCCCTGCCCTTCGCCTGGGACATCCTGTCCCCTCATTTCACCTACGGCTCCAAGGCCT
ACGTGAAGCACCCCGCCGACATCCCCGACTACTTCAAGCTGTCCTTCCCCGAGGGCTTCAAGTGGGAGCGCGTGATGAACT
ACGAGGACGGCGGCGTGGTGACCGTGACCCAGGACTCCTCCCTGCAGGACGGCGAGTTCATCTACAAGGTGAAGCTGCGC
GGCACCAACTTCCCCTCCGACGGCCCCGTGATGCAGAAGAAGACCATGGGCTGGGAGGCCTCCTCCGAGCGGATGTACCC
CGAGGACGGTGCCCTGAAGGGCAAGATCAAGATGAGGCTGAAGCTGAAGGACGGCGGCCACTACACCTCCGAGGTCAAG
ACCACCTACAAGGCCAAGAAGCCCGTGCAGCTGCCCGGCGCCTACATCGTCGACATCAAGTTGGACATCACCTCCCACAAC
GAGGACTACACCATCGTGGAACAGTACGAACGCGCCGAGGGCCGCCACTCCACCGGCGGCATGGACGAGCTGTACAAGC
ACGACGAGCTGTAACCTCAGGCACACA

Figure 2.1 Full ER-localized mOrange2 and tdTomato fluorescent protein sequence.

Individual sequence components are designated by colour. Fluorescent protein sequence only is 1431 bp for tdTomato, and 711 bp for mOrange2.

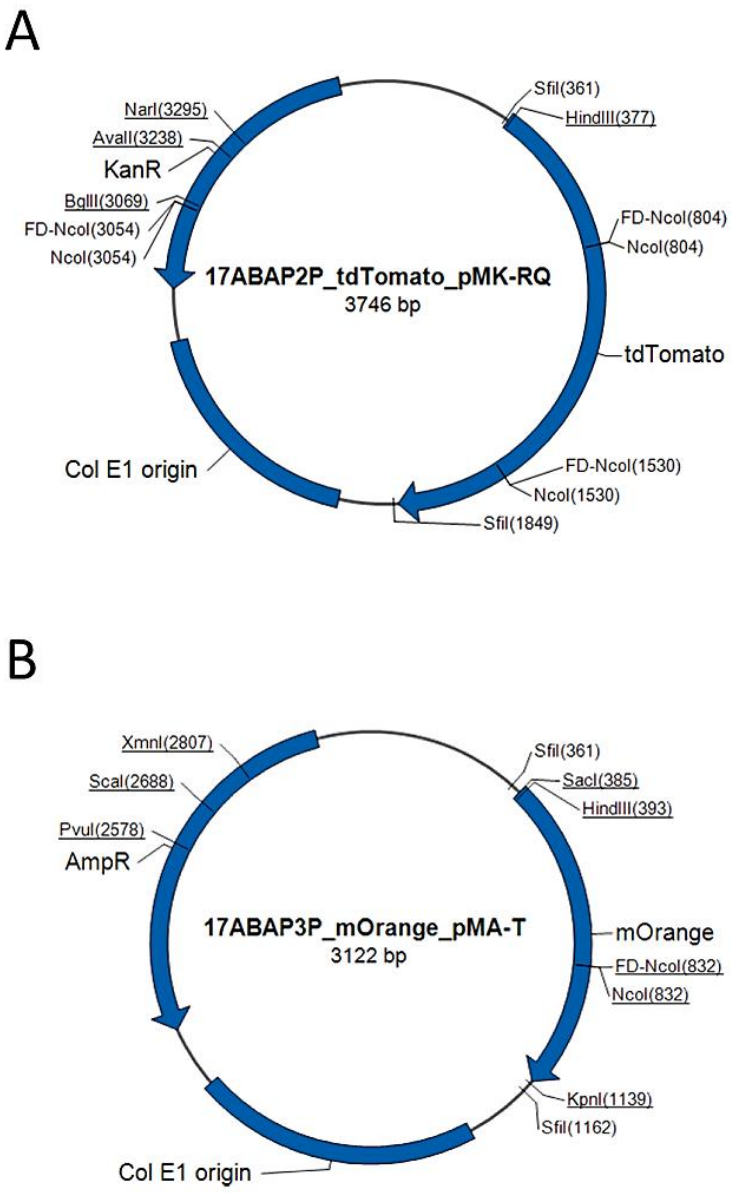

Figure 2.2 Assembly vectors containing constructed tdTomato [A] and mOrange2 [B] fluorescent protein sequences. Vectors contain kanamycin as a selectable marker in bacteria.

2.7 Cloning promoters for screening

2.7.1 Subcloning

Promoter sequences were predicted using TSSP (Shahmuradov et al., 2005) and Promoter Scan (Prestridge, 1995), both programs for predicting promoter regions in a gene. As promoters typically are found within the range of 2000-2500 base pairs upstream to the 5'UTR, 2500 base pairs prior to the 5'UTR in the genes selected for cloning are included in the analysis. Genes which did not have a predicted promoter region in either program had at least 2500 base pairs upstream of the 5'UTR included in the promoter sequences. Promoters were subcloned using the protocol and reagents in the Zero Blunt TOPO PCR cloning kit and included TOP10 strain *E. coli* (Thermo Fisher Scientific). 3 μL of fresh PCR product was used for each reaction. Primers were constructed using Primer3 (Primer3 Input version 0.4.0) using the default settings (Table 2.2).

Table 2.2 Primers of selected promoters for subcloning. Forward and reverse primers for coding sequence of selected genes are shown 5' to 3'. Full promoter sequence length in base pairs (BP) is included for accurate identification on electrophoresis gels (not shown).

Gene ID	Forward	Reverse	BP
At1g67360	AGAGGAAGCCGATGGTCAAA	TTCTCACGAACGCTAGGTGT	2695
At1g74670	TCGCCGTGAAGTAAATAACACA	TGGCCATGACTAGCTCTTGA	1990
At1g80130	TGGTTCGAGATGACCGGATT	TCTCCCGGTAATACGTGTCG	2798
At2g06850	TCGGTCGATTGGTAGTAGGC	TGCATTGTGATCATTAGGCAGA	2691
At2g40610	TGGCCTGTCTAGTCAATCCA	CGTCGTCTCCATGAGTTCCT	1976
At3g15450	GGGACTCCTTGTAAGCAGGT	ATGAGAAGCCGGACTGTTGA	2854
At3g22120	GTCCGGAAATGATTGTGGGG	CAAGCATAGGAGACGGCAAC	1315
At5g17220	CCAAGGCGGCACTAATCTTT	AGCTGCTGTTACCTGTCCAT	2747

Each solution for the PCR mix for promoter amplification was made using the protocol used with Phusion High Fidelity DNA polymerase (Thermo Fisher Scientific), and 1 μL of genomic DNA from Col-0 wildtype *Arabidopsis*. The mix was vortexed and spun down, and a MasterCycler gradient thermal cycler (Eppendorf) was used for PCR amplification. The PCR protocol for amplification of promoters for At1g67360, At2g40610, At3g15450, and At5g17220 was 98°C for 30 seconds for initial denaturation, 33 cycles of 98°C for 10 seconds, 60°C for 30 seconds, 72°C for 1:30 minutes, for denaturation, annealing, and elongating, then 72° for 10 minutes for a final elongation. The RT-PCR protocol for amplification of promoters for At1g74670, At1g80130, At2g06850, and At3g22120was 98°C for 30 seconds, 33 cycles of 98°C for 10 seconds, 63°C for 30 seconds, 72°C for 1 minute, then 72° for 10 minutes. PCR products were run at 90 volts on a 1% agarose gel.

2.7.2 Transformation of *Escherichia coli*

The destination vector pCAMBIA 1303, pCAMBIA 1303 containing fluorescent protein sequences, and final vectors with promoter and fluorescent protein were transformed into subcloning efficiency DH5α strain *E. coli* (Thermo Fisher Scientific). Each transformation consisted of a 0.5 mL microcentrifuge tube containing 50 μL of *E. coli* and 2 μL of vector mixed gently by flicking the tube then incubating on ice for 30 minutes. The cells underwent heat shock in a 42°C water bath for 20 seconds then transferred immediately to ice for 2 minutes. 250 μL of SOC medium was added to the tube and shaken at 200 rpm at 37°C for 1 hour. Cultures were plated on LB (Lysogeny broth) plates containing 50 μg/mL kanamycin to select for resistant colonies containing the vector construct.

2.7.3 Colony verification and sequencing

Before sequencing, colony PCR was used to confirm the presence of desired promoters in each vector. The colony PCR protocol for At1g67360, At2g40610, At3g15450, and At5g17220 was 95°C for 5 minutes for initial denaturation, 31 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 1:30 minutes, for denaturation, annealing, and elongating, then 72° for 10 minutes for a final elongation. The colony PCR protocol for At1g74670, At1g80130, At2g06850, and At3g22120

was 95°C for 5 minutes, 31 cycles of 94°C for 30 seconds, 62°C for 30 seconds, 72°C for 1:30 minute, then 72° for 10 minutes. PCR products were run at 90 volts on a 1% agarose gel.

Colonies containing the vector were mixed with 5 mL of LB broth containing 50 μg/mL kanamycin and shaken at 250 rpm at 37°C overnight. The protocol and reagents from the EZNA Plasmid Mini Kit I (Omega Bio-tek) were used for the extraction of plasmids from *E. coli*. and vector sequencing was conducted by Eurofins custom DNA sequencing (Eurofins Scientific, Brussels, Belgium) to confirm the correct promoter sequence.

2.8. Restriction enzyme digest and ligation

Fluorescent protein sequences and promoter sequences were cut out of their respective vectors using restriction enzymes. The sequences were purified, and PCR was used to add the desired restriction enzyme cut sites for insertion into the destination vector. tdTomato and mOrange2 sequences had cut sites designed for constitutive expression with a CaMV35S promoter already within the final destination vector or expression with the promoters chosen as potential screening genes.

Final destination vectors were constructed using restriction enzymes and ligase. The binary vector pCAMBIA 1303 (Hajdukiewicz, et al., 1994) (Figure 2.3) was chosen as the destination vector for its efficiency and availability. The destination vector pCAMBIA 1303 was cut to allow for either insertion of a fluorescent protein and a selected promoter, or for a single fluorescent protein expressed with CaMV35S. Fluorescent proteins were inserted into the destination vector prior to any insertion of promoters into same vector. All sequences were visualized and extracted from their gels, purified, and used in ligation.

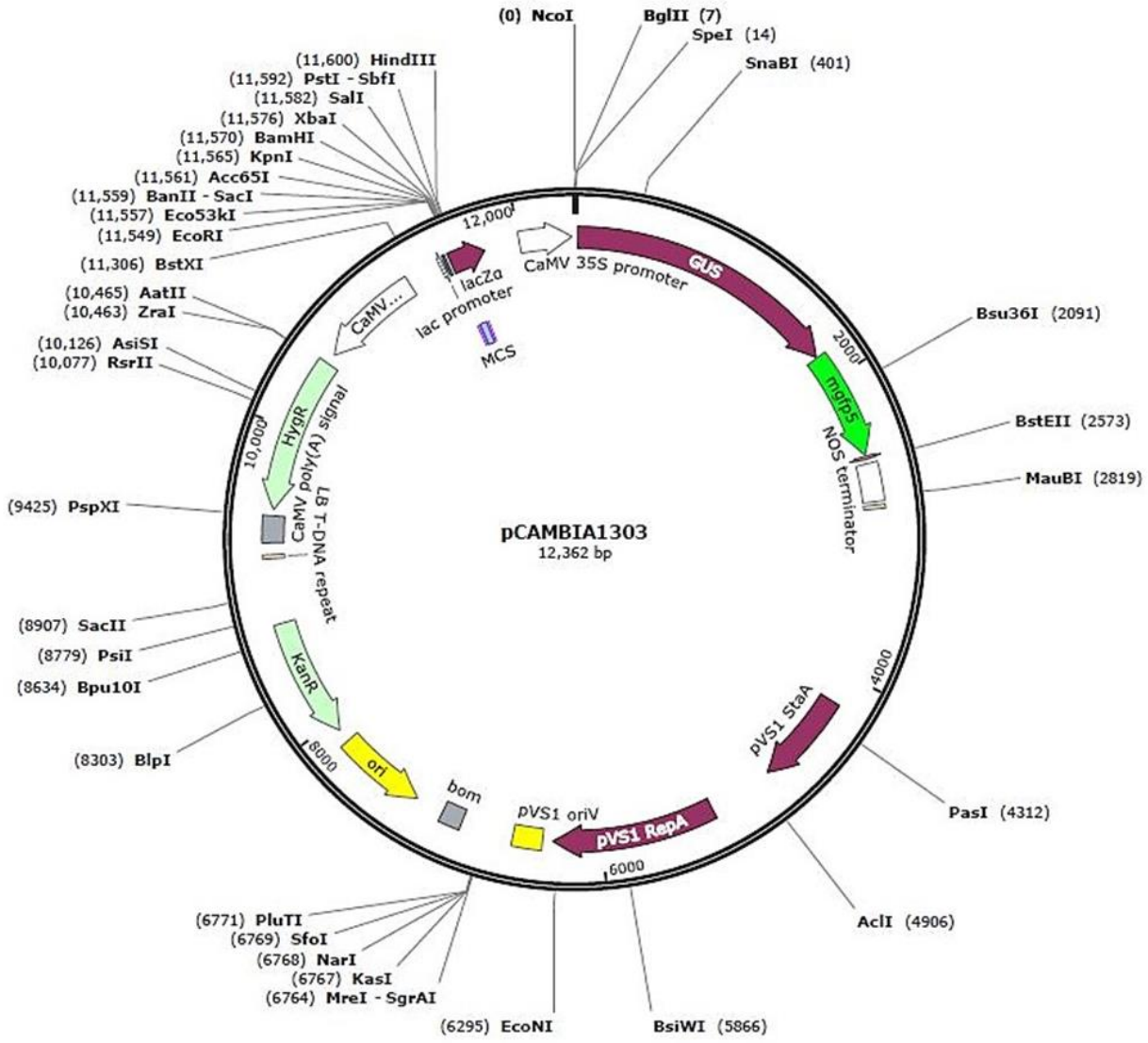

Figure 2.3 Destination vector pCAMBIA 1303. Contains a CaMV35S promoter for constitutive expression for the gene of interest.

2.8.1 Restriction enzymes and primers

Restriction enzymes were verified not to cut into any relevant sequences using RestrictionMapper (RestrictionMapper, http://www.restrictionmapper.org/). Each restriction enzyme digest for the promoters in TOPO vectors contained 1000 ng of DNA, 3 μL of EcoRI NEBuffer (New England Biolabs, Whitby, Ontario, CA), 2 μL of EcoRI, and sterile dH_2O to a total volume of 30 μL.

Restriction enzyme digests for pCAMBIA 1303 with constitutive fluorescent protein expression and their corresponding fluorescent protein sequences contained approximately 1000 ng of DNA, 4 μL of 10x Tango buffer (Thermo Fisher Scientific), 1 μL of Bsu36I, 1 μL of SpeI, and dH_2O to a total volume of 40 μL. Restriction enzyme digests for pCAMBIA 1303 without CaMV35S and their corresponding fluorescent protein sequences were the same but contained 2 μL of HindIII in place of SpeI. The same digest protocol was used to cut PCR amplified fluorescent protein sequences with restriction enzyme cut sites. Digestion reactions were undergone for 1 hour at 37°C with all enzymes before heat inactivation at 80°C for 20 minutes.

The protocol and reagents from the EZNA Gel Extraction Kit (Omega Bio-tek) were used for purifying DNA from agarose gels. Primers were made by adding restriction enzyme cut sites and a CACACA tail to previous cloning primers and fluorescent protein sequences (Table 2.3). Primers were intended to correct a single nucleotide error in the Bsu36I cut site of both fluorescent protein sequences, however no nucleotides were corrected in the primers and the fluorescent protein sequences were unaltered.

Table 2.3 Primers of selected promoters and fluorescent proteins with desired restriction enzyme cut sites. Forward and reverse primers are shown 5' to 3'. Constitutive fluorescent protein expression [FP CaMV35S expression] and fluorescent protein expression with a selected promoter [FP promoter expression] utilize the same cut site in the reverse primer.

Gene ID	Forward	Reverse
At1g74670	CACACAGAATTCTCGCCGTGAAGTAAATAACACA	CACACAGGTACCTGGCCATGACTAGCTCTTGA
At1g80130	CACACAGAATTCTGGTTCGAGATGACCGGATT	CACACAGGTACCTCTCCCGGTAATACGTGTCG
At2g06850	CACACAGAATTCTCGGTCGATTGGTAGTAGGC	CACACAGGATCCTGCATTGTGATCATTAGGCAGA
At2g40610	CACACAGAATTCTGGCCTGTCTAGTCAATCCA	CACACAGGATCCCGTCGTCTCCATGAGTTCCT
At5g17220	CACACAGAATTCCCAAGGCGGCACTAATCTTT	CACACAGGATCCAGCTGCTGTTACCTGTCCAT
FP CaMV35S expression	CACACAACTAGTATGGTGAGCAAGGGCGA	CACACACCTGAGGTTACAGCTCGTCGTGCT
FP promoter expression	CACACAAAGCTTATGGTGAGCAAGGGCGA	

Each solution for the PCR mix for promoter and fluorescent protein amplification contained 4 μL of HF Buffer, 2 μL of 2 mM dNTP mix, 0.5 μL of 20 mM forward primer, 0.5 μL of 20 mM reverse primer, 0.2 μL of Phusion High Fidelity DNA polymerase (Thermo Fisher Scientific), 5 ng of DNA, and sterile dH_2O to a final volume of 20 μL. The mix was vortexed and spun down, and a MasterCycler gradient thermal cycler (Eppendorf) was used for PCR amplification.

The PCR protocol for amplification of all promoters with restriction enzyme cut sites except At1g80130 was 94°C for 3 minutes for initial denaturation, 33 cycles of 94°C for 33 seconds, 60°C for 30 seconds, 72°C for 1:30 minutes, for denaturation, annealing, and elongating, then 72° for 10 minutes for a final elongation. The At1g80130 protocol had an annealing temperature of 62°C but all other conditions were the same. The protocol for the fluorescent proteins was the same as the promoters except with an annealing temperature of 59°C and an elongating time of 1:15 minutes. 6x DNA Gel Loading dye (Thermo Fisher Scientific) was added to the promoter PCR products and run on a 0.8% agarose gel at 90 volts to confirm product.

FastDigest enzyme digests contained 2 μL of 10x FastDigest Green buffer. 200 ng of PCR product of promoters with restriction enzyme cut sites, 1 μL of FastDigest EcoRI, 1 μL of FastDigest KpnI or 1 μL of FastDigest BamHI, and sterile dH_2O to a total volume of 30 μL. FastDigest buffer and restriction enzymes are from Thermo Fisher Scientific. Digestion of At1g74670 used FastDigest KpnI. Digestion of both At2g06850 and At2g40610 used FastDigest BamHI. Reactions containing FastDigest EcoRI were undergone for 20 minutes at 37°C, with FastDigest KpnI or FastDigest BamHI added after 15 minutes for 5 minutes, followed by heat inactivation at 80°C for 20 minutes.

2.8.2 Ligation

Fluorescent proteins were ligated into pCAMBIA 1303 followed by any promoters. Ligation was accomplished with T4 DNA ligase (Thermo Fisher Scientific) and included T4 DNA ligase buffer (Thermo Fisher Scientific). Each ligation reaction was set up on ice, consisting of 2 μL of T4 DNA ligase buffer, 1 μL of T4 DNA ligase, a 1:3 molar ratio of vector to insert, and sterile dH_2O to a final volume of 20 μL. The reaction was gently mixed by pipetting then incubated at room

temperature for 10 minutes. Heat inactivation at 65°C for 10 minutes was used to stop the reaction. The finished product was chilled on ice and used to transform *E. coli*.

2.9 *Agrobacterium*-mediated transformation of *Arabidopsis*

2.9.1 Preparation of competent cells

The gram-negative *Agrobacterium tumefaciens* was used for gene transfer into *Arabidopsis*. *Agrobacterium* was streaked on LB plates and grown overnight at 28°C. Individual colonies were inserted into 5 mL of LB broth and shaken at 250 rpm at 28°C overnight. 500 μL of this culture was aliquoted into 50 mL of LB broth and shaken at 250 rpm at 28°C for 4-6 hours until OD 600 was 0.5-1.0. 45 mL of this culture was then transferred to a cold 50 mL conical tube and kept on ice for 30 minutes. The tube was centrifuged at 4000 rpm for 10 minutes at 4°C. The supernatant was removed, the pellet was gently resuspended in cold 20 mM $CaCl_2$, and the tube centrifuged at 4000 rpm for 5 minutes at 4°C. The supernatant was once again discarded and the pellet gently resuspended in 1 mL of cold 20 mM $CaCl_2$. 250 μL of cells in suspension were aliquoted to pre-chilled 1.5 mL tubes for use in transformation.

2.9.2 Transformation of *Agrobacterium*

250 μL of *Agrobacterium* and 10 μL of destination vector were gently mixed by tapping the side of the tube then put on ice for 30 minutes. The cells then underwent heat shock in a 37°C water bath for 5 minutes. They were then put on ice for 5 minutes. After 5 minutes 1 mL of LB was added to the reaction and shaken for 2-3 hours at 28°C. The culture was plated on LB plates with 50 μg/mL kanamycin and grown for 2 days at 28°C.

2.9.3 *Agrobacterium* floral dip

Individual colonies of *Agrobacterium* were inserted into 5 mL of LB broth containing 50 μg/mL of kanamycin and shaken at 250 rpm at 28°C overnight. 2 mL of this culture was then aliquoted into 200 mL of LB broth containing 50 μg/mL kanamycin and shaken at 250 rpm at 28°C overnight. The culture was aliquoted into 50 mL conical tubes and centrifuged at 4500 rpm for 10

minutes at 18°C. The supernatant was removed and the cells were gently resuspended in 200 mL of 5% W/V sucrose solution. Silwet-77 was added to a final concentration of 0.05% V/V. Several days before transformation *Arabidopsis* bolts were clipped to encourage secondary inflorescences. Inflorescences were dipped in *Agrobacterium* solution and gently agitated for 2-3 seconds. Plants were lain on their side and covered for 1 day at room temperature. They were then uncovered and moved to a growth chamber following a 16-hour day protocol.

2.9.4 Identifying transformed plants

Transformed plants were watered for a week then allowed to dry. Seeds were collected then incubated for two days at 4°C. Seeds were surface sterilized then plated on 25 μg/mL hygromycin MS salt with nitrogen (PhytoTechnology Labs, Kansas, USA) plates containing 2.17 g of MS salt , 5 g of sucrose, and 8 g of agar per 1 L of dH_2O.

T_1 seedlings were transferred to soil in a pot and moved to a growth chamber following a long-day cycle (16 hours 122 μM of photons m^{-2} s^{-1}; 8 hours dark) at a relative humidity of 65% and 22°C. DNA from shoot tissue was extracted following the protocol by Edwards, Johnstone, & Thompson (1991). PCR was conducted using shoot tissue DNA with FP CaMV35S expression forward and reverse primers to confirm presence of promoter-marker construct. The PCR protocol and primers are identical to those used for cloning.

3. RESULTS

3.1 RNA-Seq analysis

RNA-Seq data from control and clubroot inoculated *Arabidopsis* shoot tissue was used to select potential reliable reporter genes for the detection of early clubroot infection. Parameters of selection were based on gene expression patterns that exhibit desired reporter gene traits. Data was available for 17, 20, and 21 DPI, however only changes in expression on 20 DPI and 24 DPI were considered during gene selection. This is because 17 DPI is considered pre-infection and limited differential expression was expected at this time point. Expression of genes in the RNA-Seq data is expressed in Fragments Per Kilobase per Million (FPKM) units. FPKM takes into account the sequencing depth and gene length and normalizes them by dividing the total reads by a million, dividing individual read counts by "per million" total reads, and then dividing that value by the length of the gene. Hereafter, normalized FPKM units will be referred to as "reads". Control 20 DPI and 24 DPI were within 200 reads of each other, while clubroot inoculated 20 DPI and 24 DPI were within 500 reads of each other. The expression between control and inoculated in both 20 DPI and 24 DPI were different by at least a factor of two. Lastly, between both 20 DPI (control and inoculated) and 24 DPI (control and inoculated) there was a difference greater than 100 reads. These parameters yielded 19 potential reporter genes (PRGs) among hundreds. All PRG expression levels in both control and inoculated samples averaged between 0.17 and 787 reads.

Genes for potential clubroot infection reporters were grouped into four categories based on their expression: Category 1, up-regulation in inoculated plants under 500 total reads; Category 2, up-regulation in inoculated plants under 800 total reads; Category 3, down-regulation in inoculated plants under 300 total reads; Category 4, down-regulation in inoculated plants under 500 total reads.

These categories were devised to illustrate the relative change in gene expression between genes in control and inoculated plants on 17, 20, and 24 DPI. They may also provide insight into the ideal level of expression for a reporter gene. A PRG tag with the category number followed by a letter designation within the category was assigned for the ease and clarity of discussing genes without referring to their gene ID. Functions for PRGs (Table 3.1) were obtained from The Arabidopsis Information Resource (TAIR, Phoenix Bioinformatics TAIR).

Table 3.1 Potential reporter gene ID, gene function, and PRG tag. PRGs were assigned a category based on their expression pattern in clubroot infected *Arabidopsis* shoot tissue and assigned a letter to identify them within their category.

Gene ID	Function	PRG Tag
At1g27020	Domain of unknown function 1338	1a
At3g44860	Farnesoic acid carboxyl-O-methyltransferase, *FAMT*	1b
At4g14090	Anthocyanin 5-O-glucosyltransferase, *UGT75C1*	1c
At5g13930	Chalcone synthase, *CHS*	1d
At5g17220	Glutathione s-transferase 26, *GST26*	1e
At5g42800	Dihydroflavonol 4-reductase, *DFRA*	1f
At1g64360	Senescence associated and QQS (Qua Quine Starch) related, *SAQR*	2a
At1g67360	Lipid droplet associated protein 1, *LDAP1*	2b
At1g80130	TPR (tetratricopeptide repeat) -like superfamily protein	2c
At5g20190	TPR (tetratricopeptide repeat) -like superfamily protein	2d
At1g69530	Expansin A1, *EXP1*, alpha-expansin family	3a
At1g74670	Gibberellin-stimulated *Arabidopsis* 6, *GASA6*	3b
At2g06850	Endoxyloglucan transferase, *EXGT-A1*	3c
At2g22990	Sinapoylglucose malate sinapoyltransferase, *SNG1*	3d
At2g40610	Expansin A8, *EXP8*, alpha-expansin family	3e
At3g13750	Beta galactosidase 1, *BGAL1*	3f
At3g15450	Aluminum induced protein with YGL and LRDR motifs, *AILP1*	3g
At3g22120	Cell wall-plasma membrane linker protein, *CWLP*	4a
At5g24490	Plastid-specific ribosomal protein 1	4b

The change in expression of up-regulated PRGs in inoculated plants on DPI 20 and DPI 24 was generally relatively higher than the change in expression of down-regulated PRGs in inoculated plants. Assuming the rate of transcript turnover is similar in both up- and down-regulated PRGs, it was possible that up-regulated PRGs were differentially expressed earlier during infection. However, a number of other explanations could account for this disparity: up-regulated genes may have had a higher transcription rate when activated or down-regulated genes may have higher transcript turnover than up-regulated genes.

The known functions of Category 1 PRGs were either related to plant defense mechanisms (Figure 3.1 1b; 1e) or stress responses (Figure 3.1 1c; 1d; 1e; 1f). An increase in expression of defense and stress associated genes can be anticipated during any kind of stress and may not have the reporter specificity for clubroot detection. Category 2 PRGs were signaling genes up-regulated during stress. Many Category 3 PRGs were involved in cell wall function or cell proliferation. Overall, 5 out of 19 PRGs (Figure 3.3 3a; 3c; 3e; 3f, Figure 3.4 4a) showed some association with the plasma membrane or the cell wall and all were down-regulated following infection. Category 4 PRGs were either involved in signaling or chloroplast synthesis.

Almost all genes in categories that showed the largest relative changes in gene expression were either involved in cell membrane function (PRGs 2c, 2d, 4a), cell death (PRG 2a), or protein synthesis (PRG 4b). There might be changes in expression which are disease specific in the cell membrane related genes, as clubroot infection results in highly modified cell walls, or they may play large roles in overall cell maintenance.

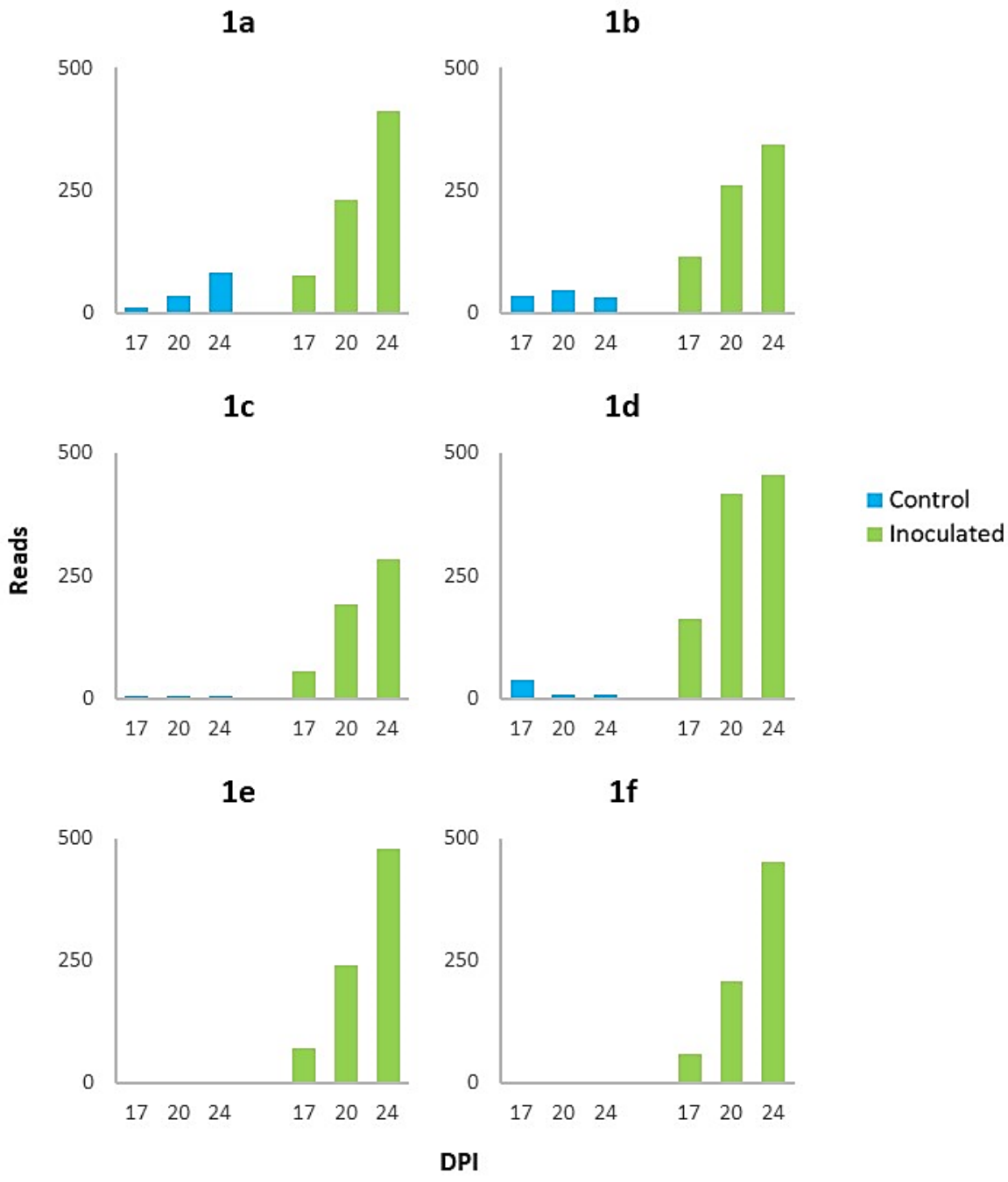

Figure 3.1 RNA-Seq data displaying Category 1 PRG expression in control and clubroot inoculated *Arabidopsis* shoot tissue on 17, 20, and 24 DPI. PRGS are designated by their PRG tags. Control expression is in blue, inoculated expression is in green.

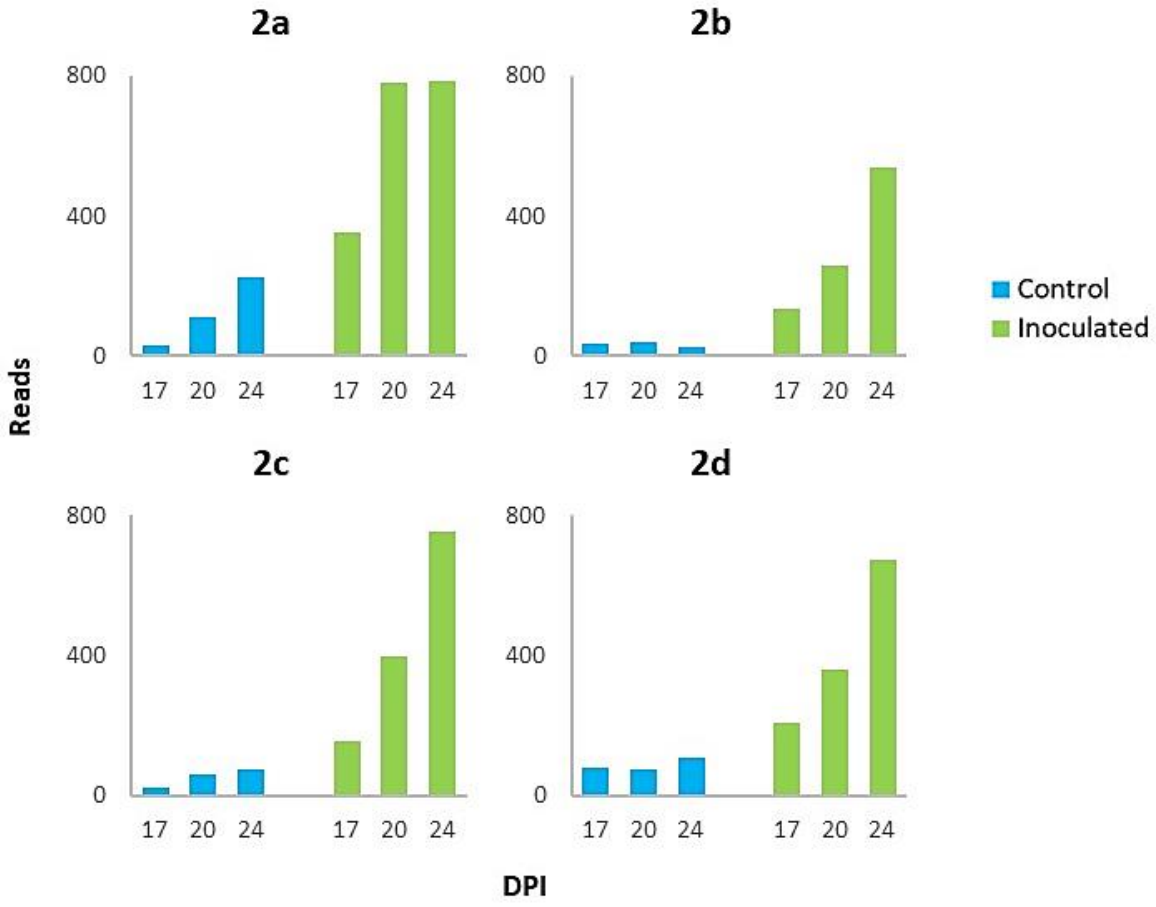

Figure 3.2 RNA-Seq data displaying Category 2 PRG expression in control and clubroot inoculated *Arabidopsis* shoot tissue on 17, 20, and 24 DPI. PRGS are designated by their PRG tags. Control expression is in blue, inoculated expression is in green.

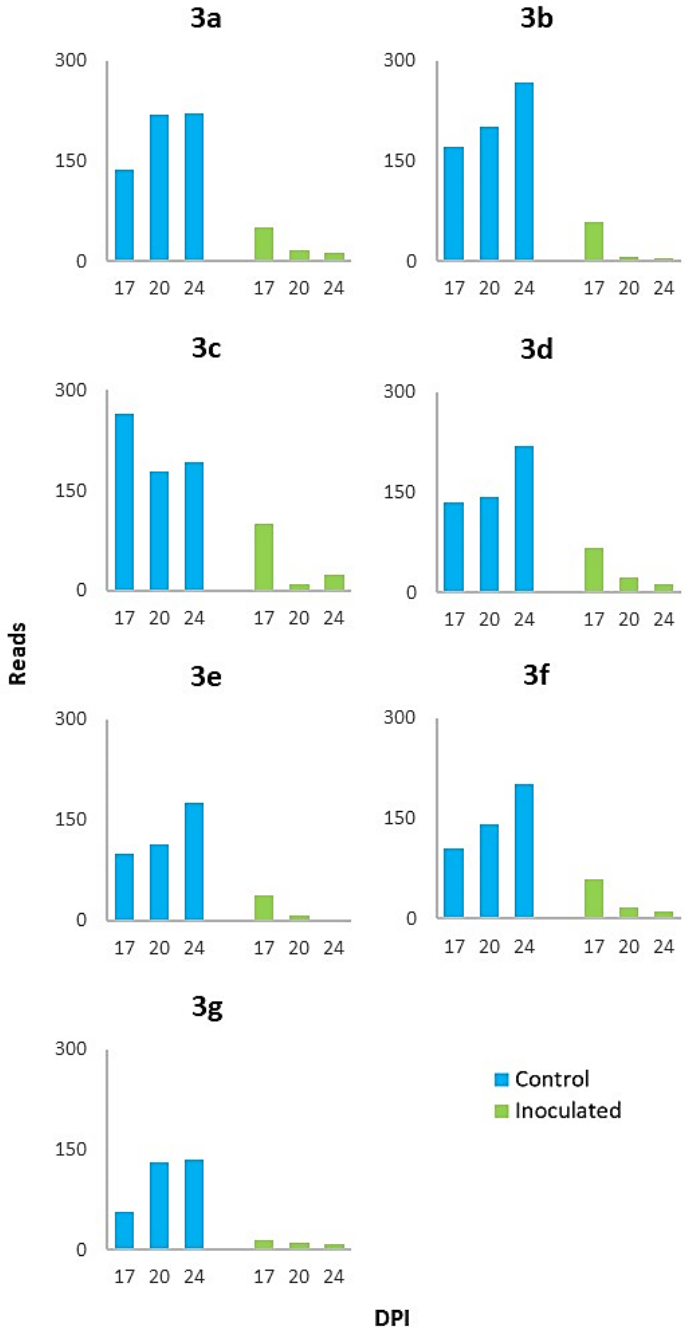

Figure 3.3 RNA-Seq data displaying Category 3 PRG expression in control and clubroot inoculated *Arabidopsis* shoot tissue on 17, 20, and 24 DPI. PRGS are designated by their PRG tags. Control expression is in blue, inoculated expression is in green.

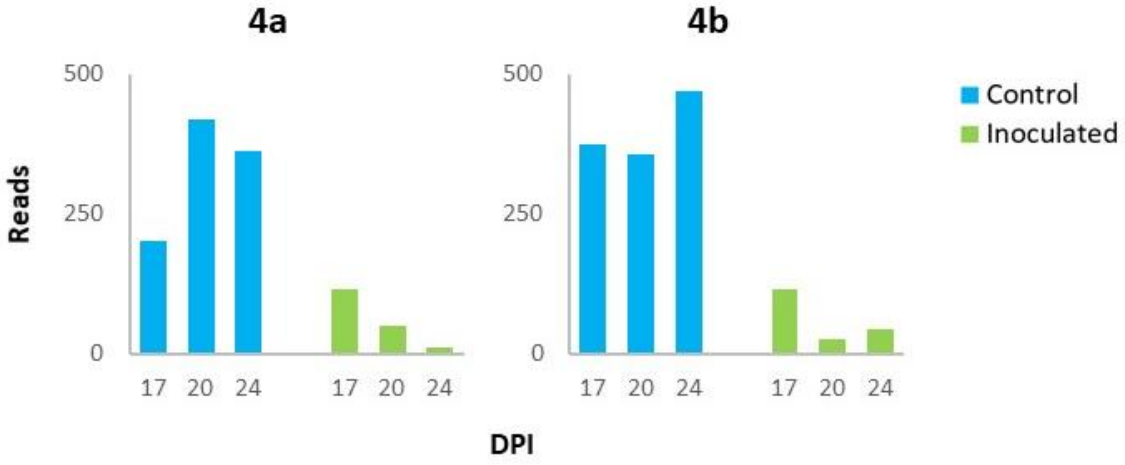

Figure 3.4 RNA-Seq data displaying Category 4 PRG expression in control and clubroot inoculated *Arabidopsis* shoot tissue on 17, 20, and 24 DPI. PRGS are designated by their PRG tags. Control expression is in blue, inoculated expression is in green.

3.2 Infection phenotype

RNA-Seq data showed that the most discernable changes in gene expression during infection occurred sometime after 17 DPI. A time course for 0, 2, 5, 7, 14, 21, and 28 DPI was used to track clubroot infection. Inoculated plants appeared slightly smaller than the control by DPI 12 but are likely indistinguishable within larger mixed sample groups. By DPI 21 there were distinct phenotypical symptoms of clubroot infection in inoculated plants; shoots were visibly smaller and stressed, and the root was truncated and swollen with a well-defined gall (Figure 5). On 12 DPI (Figure 5; A) there was a very slight difference in shoot size between control and inoculated plants, with control plants being minutely larger. This may be because infection was not yet severe enough by 12 DPI to alter gene activity in the shoot tissue, despite a visible decrease in fresh weight yield. By 21 DPI there was an obvious difference between infected and control shoots (Figure 5; B) with visible gall formation in the root (Figure 5; C). Infected shoots were smaller and exhibited purpling in the petioles, indicating the build-up of anthocyanin. Infected roots were truncated and had fewer lateral roots. Once visual signs of clubroot infection were present in the shoot, there was a small window of time wherein a large amount of phenotypic variation was observed.

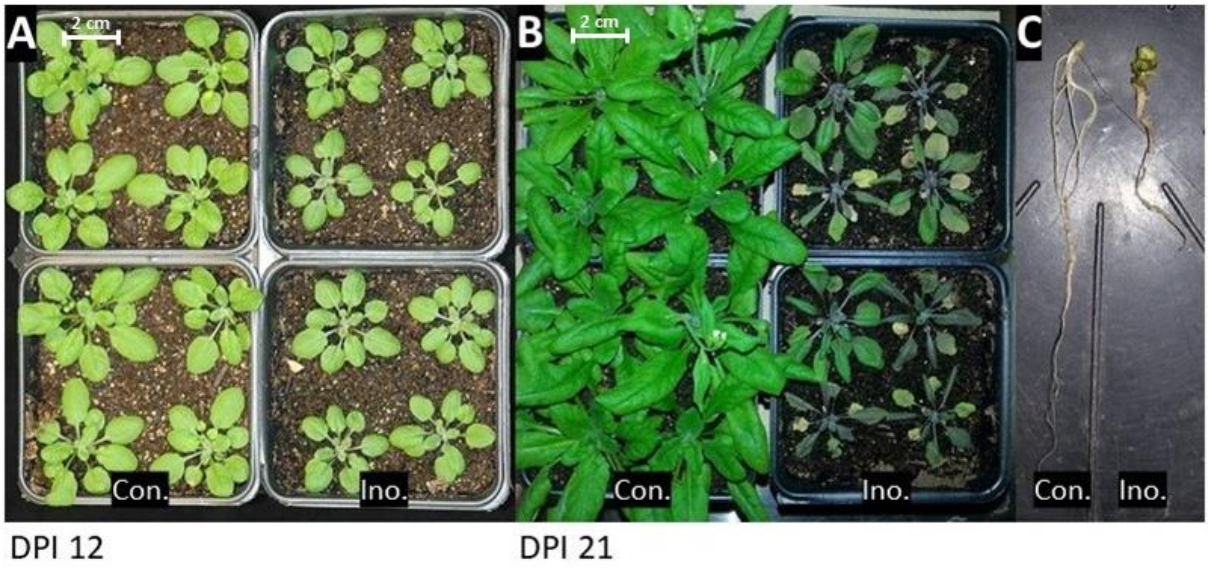

Figure 3.5 Control [Con.] and clubroot inoculated [Ino.] *Arabidopsis* shoots.
Representative image of Arabidopsis shoots 12 DPI [A], as well as shoots [B] and roots [C] 21 DPI.

3.3 RT-PCR expression

RNA-Seq data trends and phenotypic time course observations were further investigated using RT-PCR. Interestingly, some PRGs in Category 1 PRGs (Figure 3.6 1a; 1c; 1d; 1e; 1f) in inoculated plants maintained steady expression during disease progression with down-regulation seen in control plants. PRG 1c maintained relatively steady expression throughout the time course in inoculated plants but was down-regulated on 14, 21, and 28 DPI in control plants. A similar trend was seen in PRG 1d, however there was no down-regulation in the control plants until 28 DPI. No change in expression throughout disease progression suggests these PRGs may be unreliable reporters. PRG 1a was differentially expressed post 5 DPI, however displayed the same expression post 14 DPI. This may also indicate poor reporter gene qualities. PRGs 1b (Figure 3.6 1b) and 1f exhibited a slight down-regulation in inoculated plants on 21 and 28 DPI. PRG 1e appeared to be slightly up-regulated on 21 and 28 DPI.

Category 2 PRGs generally displayed differential expression at DPI 21 and 28 (Figure 3.7). Most were down-regulated (Figure 3.7 2a; 2b; 2d) in the RT-PCR in inoculated plants which is in opposition to what was seen in the RNA-Seq. PRG 2c (Figure 3.7 2c) was seen to be up-regulated in inoculated plants DPI 5 onwards, with no expression in control plants throughout the time course. As PRG 2c is only expressed in inoculated plants, it may indicate an inoculation-specific gene. This specificity would be advantageous in a reporter gene.

Category 3 PRGs (Figure 3.8 3a; 3b; 3c; 3e; 3f; 3g) generally displayed down-regulation on DPI 21 and 28 in inoculated plants, consistent with the RNA-Seq data, with the exception of PRG 3d (Figure 3.8 3d), which was slightly up-regulated on those DPI.

Category 4 PRGs (Figure 3.9) expression trends were consistent with RNA-Seq data, with down-regulation in inoculated plants on DPI 21 and 28.

In general, RT-PCR results post 14 DPI in conjunction with developing phenotypic differences at 12 DPI and obvious phenotypic differences at 21 DPI suggests that the best reporter for early infection will show changes in gene expression after 14 DPI and before 21 DPI. This

intermediate window of time was unfortunately not included in the time course, as the time course was based on concurrent research on *Plasmodiophora*.

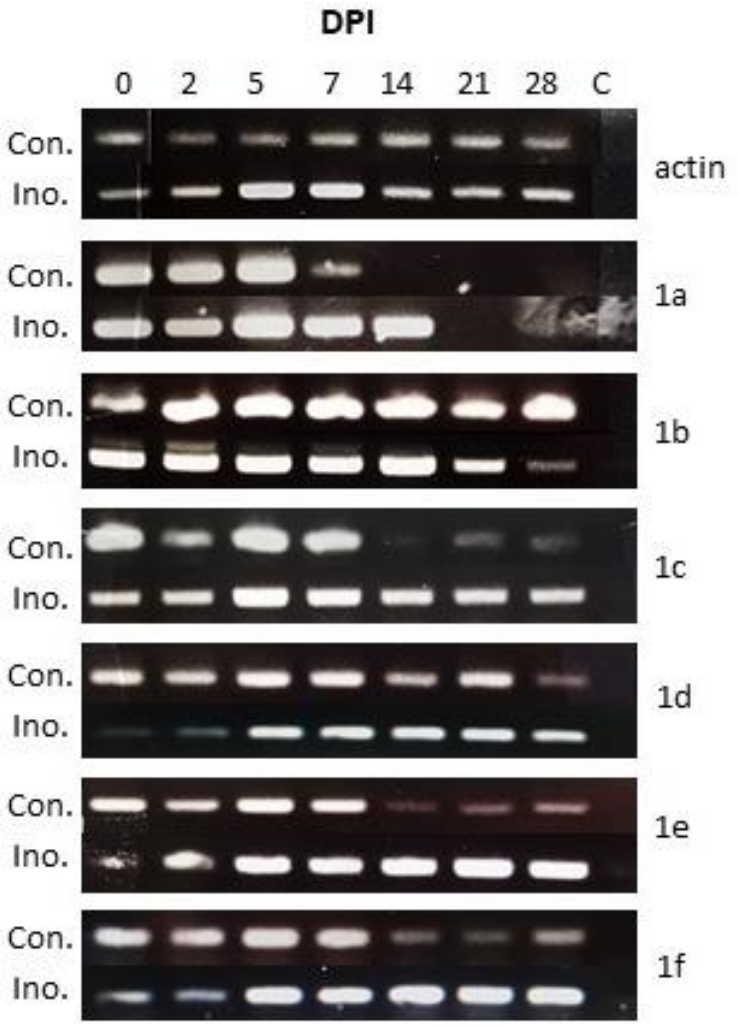

Figure 3.6 RT-PCR of Category 1 PRG expression in control [Con.] and clubroot inoculated [Ino.] *Arabidopsis* shoot tissue on 0, 2, 5, 7, 14, 21, and 28 DPI. Image is representative of RT-PCR reactions from 3 separate replicates. Autoclaved H_2O was used as a negative control.

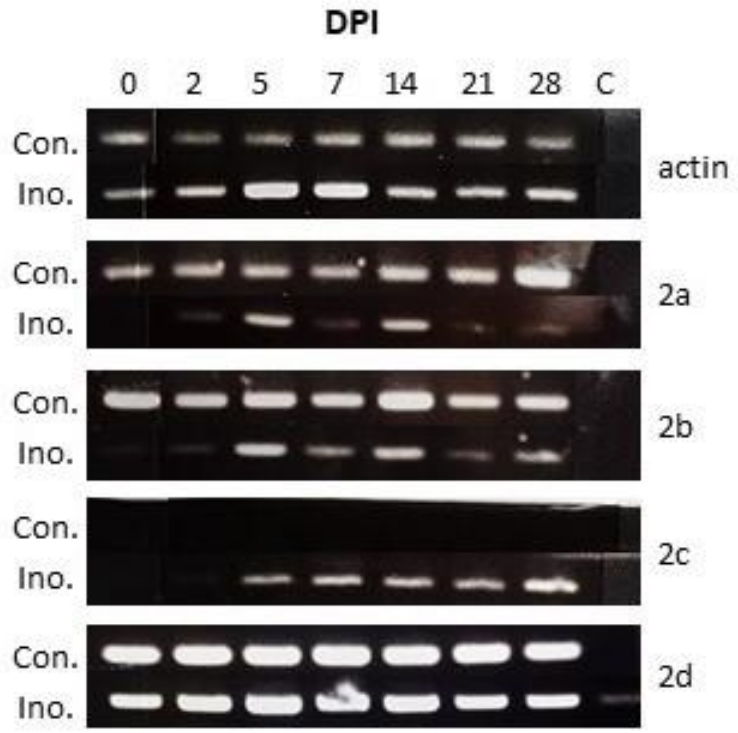

Figure 3.7 RT-PCR of Category 2 PRG expression in control [Con.] and clubroot inoculated [Ino.] *Arabidopsis* shoot tissue on 0, 2, 5, 7, 14, 21, and 28 DPI. Image is representative of RT-PCR reactions from 3 separate replicates.

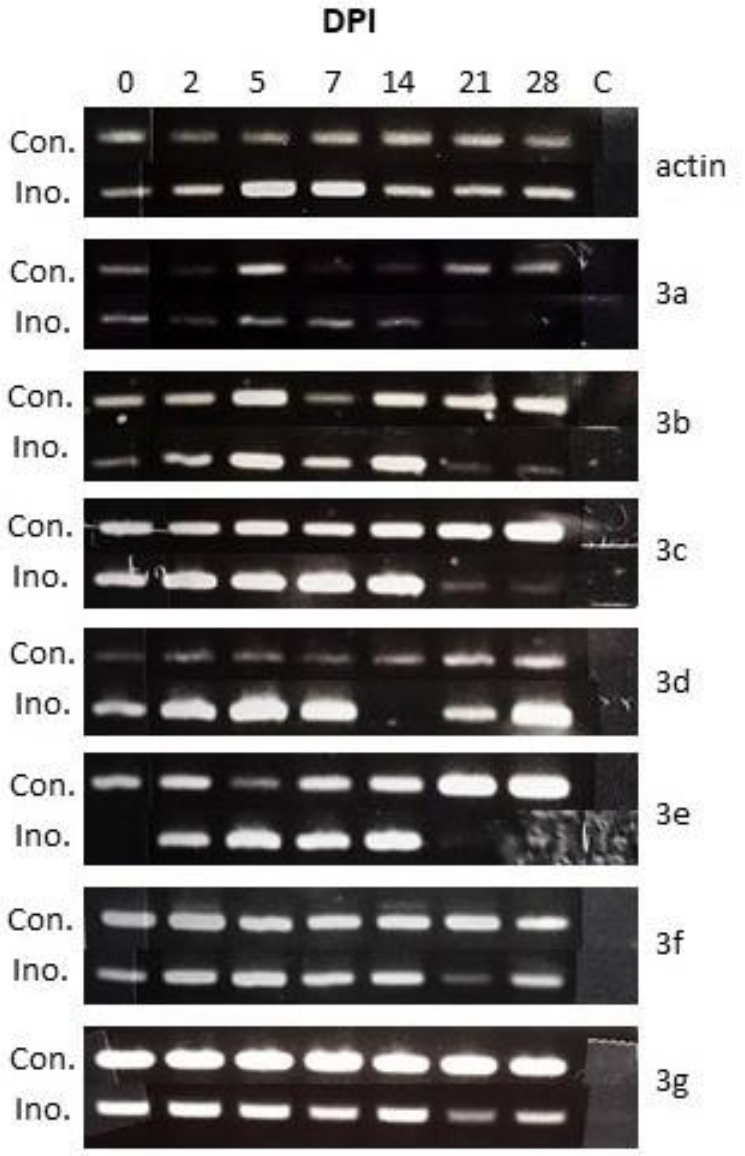

Figure 3.8 RT-PCR of Category 3 PRG expression in control [Con.] and clubroot inoculated [Ino.] *Arabidopsis* shoot tissue on 0, 2, 5, 7, 14, 21, and 28 DPI. Image is representative of RT-PCR reactions from 3 separate replicates. Autoclaved H_2O was used as a negative control.

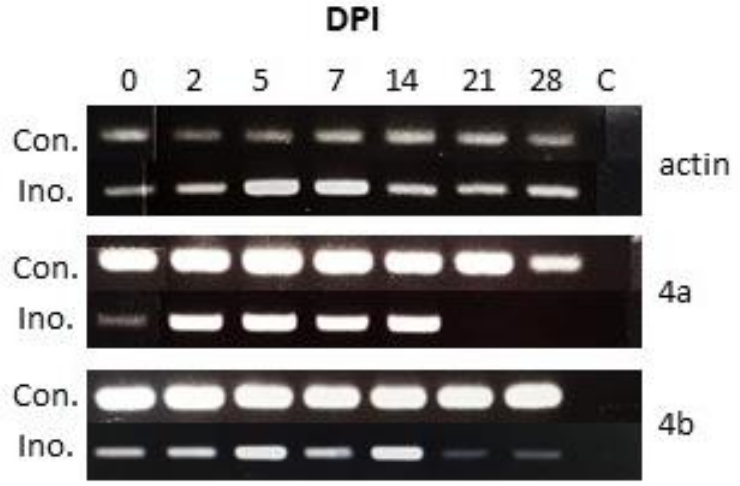

Figure 3.9 RT-PCR of Category 4 PRG expression in control [Con.] and clubroot inoculated [Ino.] *Arabidopsis* shoot tissue on 0, 2, 5, 7, 14, 21, and 28 DPI. Image is representative of RT-PCR reactions from 3 separate replicates. Autoclaved H_2O was used as a negative control.

3.4 RT-qPCR expression

Based on similar trends in the RNA-Seq, RT-PCR, and one initial RT-qPCR reaction (data not shown), 8 PRGs out of the initial 19 were chosen for RT-qPCR analysis. The ΔΔCt method (Livak & Schmittgen, 2001) was used to analyze three replications of RT-qPCR data from the same time course samples used for RT-PCR. A positive ΔΔCt value indicated an increase in gene expression in the inoculated shoot tissue. Conversely a negative ΔΔCt value indicated a decrease in gene expression in the inoculated shoot tissue.

There was a trend of down-regulation in PRG 1e (Figure 3.10) as disease progressed. This was not consistent with the up-regulation of PRG 1e in inoculated plants compared to control plants seen in the RNA-Seq and RT-PCR. PRG 2b (Figure 3.11 2b) did not display any observable trend between DPI. PRG 2c (Figure 3.11 2c) appeared to be slightly up-regulated and then down-regulated as infection progressed, however the trend did not appear conspicuous. There was an up-regulation on 28 DPI in PRG 2c which was also seen in the RNA-Seq and RT-PCR. PRGs 3b and 3e (Figure 3.12 3b; 3e) displayed no obvious trend between 0 and 14 DPI, then displayed a slight up-regulation in expression on 21 DPI. PRG 3c (Figure 3.12 3c) appeared to be up-regulated on 21 and 28 DPI, however the error bars on these DPI were fairly large and thus is it unlikely any trend is present. PRG 3g (Figure 3.12 3g) is initially down-regulated then up-regulated by 5 DPI. PRG 4a (Figure 3.13) was consistently down-regulated, which was consistent with the RNA-Seq and RT-PCR trends.

A notable trend found in most of the PRGs (Figure 3.11, Figure 3.12 3b; 3e, Figure 3.13) was the drastic difference between control and inoculated plants on DPI 28 compared to other DPI. This was likely due to cellular damage to the point where RNA extraction was not viable. A positive or negative value on 0 DPI was likely due to difference in cDNA quality between control and infected plants, as samples were small and yielded minimal RNA.

Using one-way ANOVA ($p=0.05$) none of the PRGs were shown to have a significant difference in expression between the days post inoculation observed. This may be because there truly is no significant difference, or it may be due to a high standard error among samples. The one-way ANOVA was also conducted under the assumption that actual days post infection

corresponded directly to days post inoculation for every replication, however there was no way to confirm whether that was the case. In the case that there is no significant difference, the RT-PCR results could be an exaggeration of the difference in gene presence or lack thereof.

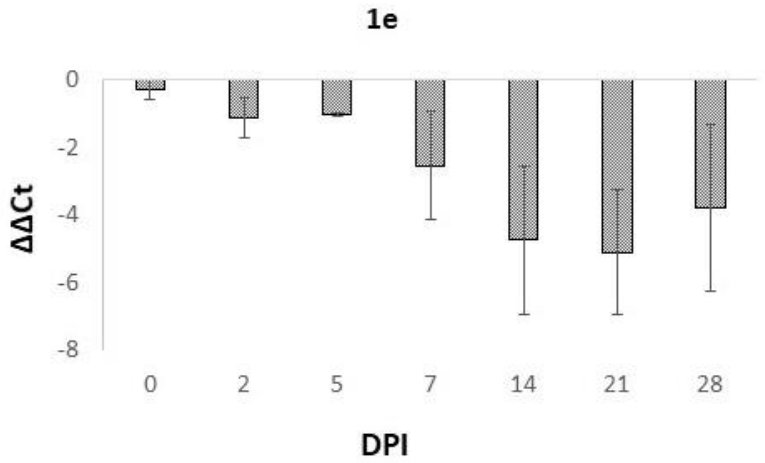

Figure 3.10 RT-qPCR data displaying differential expression of PRG 1e in clubroot inoculated *Arabidopsis* shoot tissue as ΔΔCt on 0, 2, 5, 7, 14, 21, and 28 DPI. There was no significant difference in PRG expression between DPI (p=0.05).

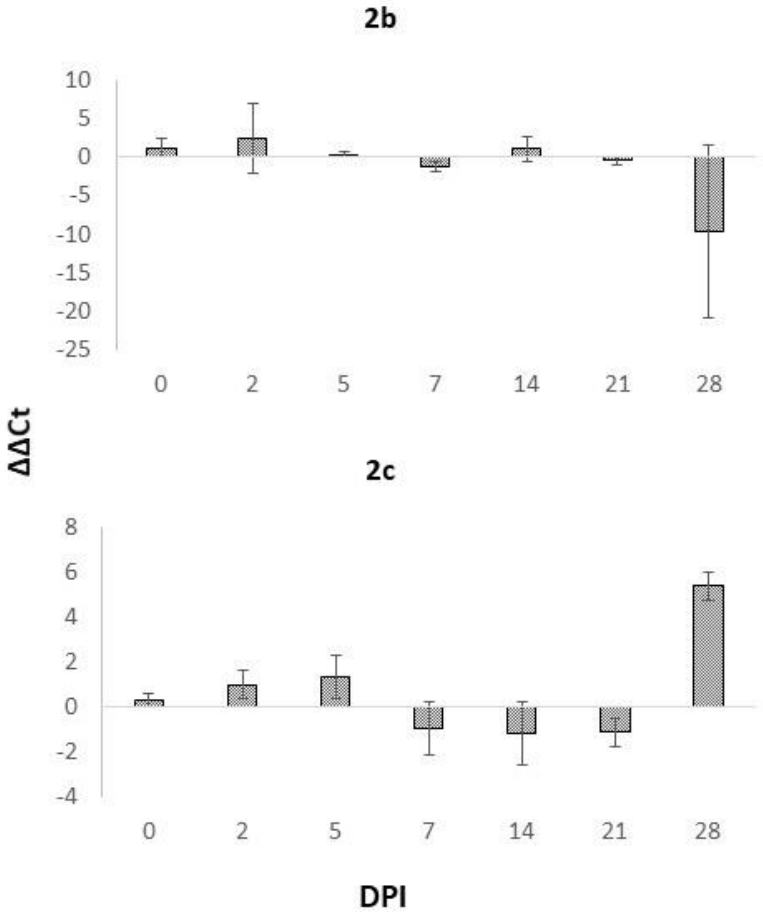

Figure 3.11 RT-qPCR data displaying differential expression of PRGs 2b and 2c in clubroot inoculated *Arabidopsis* shoot tissue as ΔΔCt on 0, 2, 5, 7, 14, 21, and 28 DPI. There was no significant difference in PRG expression between DPI (p=0.05).

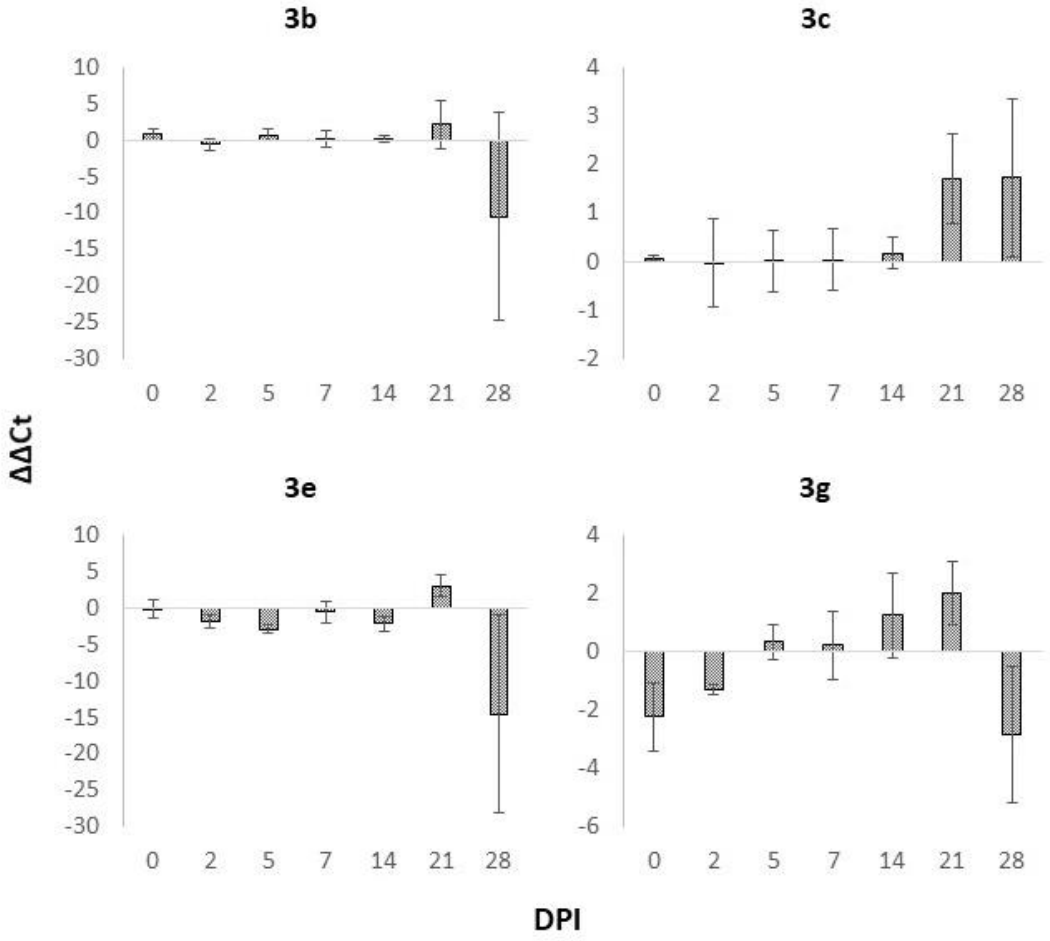

Figure 3.12 RT-qPCR data displaying differential expression of PRGs 3b, 3c, 3e, and 3g in clubroot inoculated *Arabidopsis* shoot tissue as ΔΔCt on 0, 2, 5, 7, 14, 21, and 28 DPI. There was no significant difference in PRG expression between DPI (p=0.05).

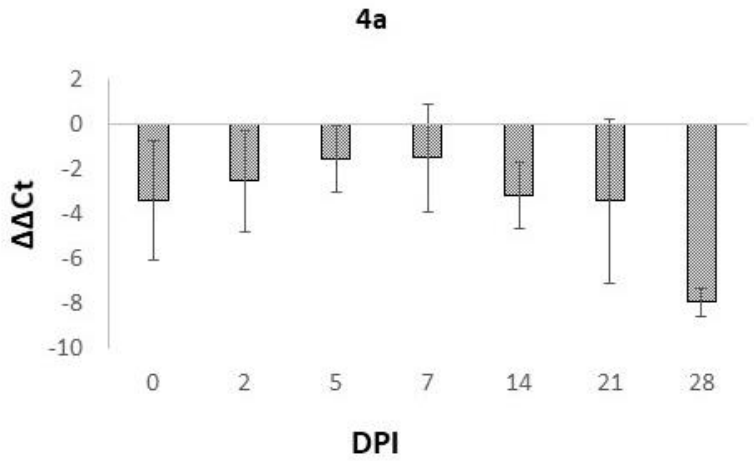

Figure 3.13 RT-qPCR data displaying differential expression of PRG 4a in clubroot inoculated *Arabidopsis* shoot tissue as ΔΔCt on 0, 2, 5, 7, 14, 21, and 28 DPI. There was no significant difference in PRG expression between DPI (p=0.05).

3.5 Constructed promoter-reporter vectors

The 8 PRGs selected based on RNA-Seq, RT-PCR, and one replication of RT-qPCR results were used to construct promoter-reporter vectors. Cloning occurred concurrent to RT-qPCR analysis due to time restraints. PRG promoters were subcloned into TOPO vectors and transformed into *E. coli*. TOPO vectors were extracted from *E. coli* and promoters for PRGs 1e, 2c, 3b, 3c, and 3e were successfully isolated using restriction enzymes. PCR was used to add restriction enzyme cut sites to the purified PRG 3b, 3c, and 3e promoters for ligation into the destination vector pCAMBIA 1303. Endoplasmic reticulum retention fluorescent protein sequences for tdTomato and mOrange2 were designed and then cut out of their assembly vectors using restriction enzymes. PCR was used to add restriction enzyme cut sites to purified fluorescent protein sequences for activation with a PRG promoter or constitutive expression with a CaMV promoter. PRG promoters and either tdTomato or mOrange2 were ligated into pCAMBIA 1303 and transformed into *Agrobacterium*. A promoter-reporter vector containing the PRG 3b promoter and mOrange2 fluorescent protein was constructed and introduced into *Arabidopsis* via *Agrobacterium*-mediated transformation.

3.6 *Arabidopsis* transgenic lines

Transformation of *Arabidopsis* with *Agrobacterium* containing mOrange2 expression with a *Gibberellin stimulated Arabidopsis* 6 (PRG 3b) promoter (*GASA6::mOrange2*) was successful. Multiple transformations with individual hygromycin resistant *Agrobacterium* colonies were attempted, with a minimum of two plates for each. Among transformed seedlings grown on a hygromycin plate (Figure 3.14), seedlings which appeared to be larger than others were selected as possible transgene carriers and grown in soil. RT-PCR of genomic DNA from these seedlings confirmed the presence of the mOrange2 gene (Figure 3.15). Two T1 transgenic lines were established and designated *GASA6::mOrange2*-ginger1 (ginger1) and *GASA6::mOrange2*-ginger2 (ginger2).

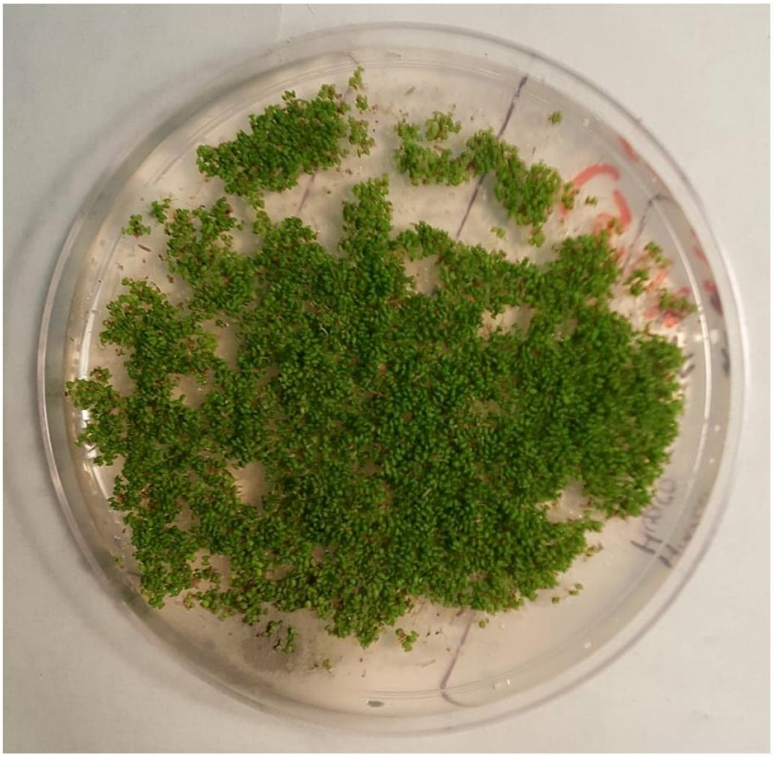

Figure 3.14 *Arabidopsis* seedlings at 14 days old on a 25 µg/mL hygromycin MS plate.

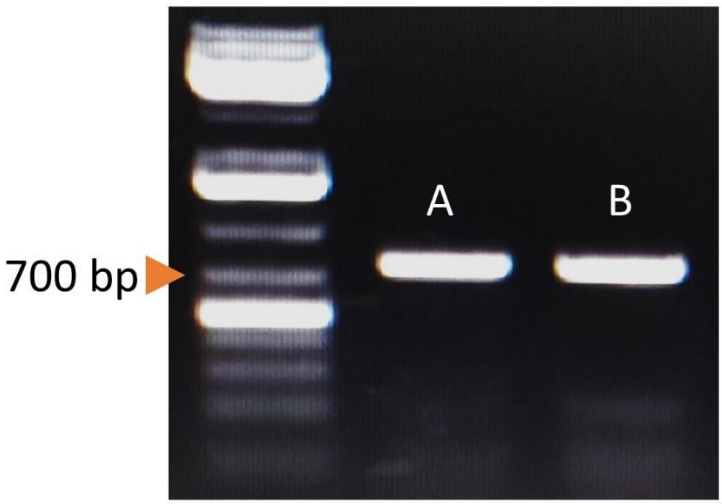

Figure 3.15 Genomic mOrange2 presence in seedling shoots in T_1 transgenic lines ginger1 [A] and ginger2 [B]. Pictured with 1kb+ ladder.

4. DISCUSSION

4.1 Project objectives

To address the difficulty of detecting early infection of clubroot disease, the goal of my research was to identify potential genes whose change in expression pattern would act as an indicator of early clubroot infection in above-ground tissue using a non-destructive fluorescence-based method. My first objective was achieved by analyzing RNA-Seq data from clubroot infected *Arabidopsis* shoots (Irani et al., 2018). Parameters for gene selection were optimized to obtain genes that would act as reliable reporters. As there is generally no phenotypic variation in the shoots until 17 DPI with comparatively minimal difference in expression between control and infected plants, changes in gene expression were focused on 20 DPI and 24 DPI. This also provided a larger pool of PRG candidates, as Irani et al. (2018) found only 1445 differentially expressed genes in inoculated shoot tissue on 17 DPI compared to the 6784 on 20 DPI and 8639 on 24 DPI.

In the chosen genes control 20 DPI and control 24 DPI were within 200 reads of each other, to maintain consistency in expression within control plants, as their expression should not change dramatically within these five days. Inoculated 20 DPI and inoculated 24 DPI were within 500 reads of each other, also to account for consistency in expression while allowing for the potential of greater gene expression variability in inoculated plants which may not be present in control plants. Between both 20 DPI (control and inoculated) and 24 DPI (control and inoculated) there was a difference greater than 100 reads. A substantial difference in expression was required to differentiate between increased or decreased expression in a visual capacity, as seen with RT-PCR gels and induced fluorescence. For this same reason, expression between control and inoculated (20 and 24 DPI) were different by at least a factor of two ($\geq$100 reads). Parameters were refined until

these final parameters yielded 19 potential gene candidates (Table 3.1), which was deemed a practical number of genes to examine at the initial stages of this project. The categorization of these genes based on expression pattern and level of expression provided an efficient way to discuss the expression trends of PRGs without referring back to specific gene names or IDs.

My second and third objectives were completed by using a time-course following infection with clubroot spores to investigate gene expression during disease progression. Time points at 0, 2, 5, 7, 14, 21, and 28 DPI were selected to harvest inoculated and control shoot tissue. These time points were selected based on concurrent research at the same DPI time points by my colleagues in the department focusing on the clubroot parasite Plasmodiophora brassicae in the host. This would give us the option to compare changes in gene expression in both host and parasite at the same stage of infection. Time points 0, 2, 5, 7, and 14 DPI are also well before infection is noticeably visible in shoots and changes in gene expression due to infection during this time could indicate a marker for early infection. One replication of RT-PCR and RT-qPCR results for the expression of PRGs were compared to RNA-Seq data, and 8 PRGs (see: section 4.1) that displayed the same initial expression trend in all three tests were selected for cloning and further investigation concurrent with further RT-qPCR analyses.

RT-PCR results that did not coincide with RNA-Seq trends included PRGs 1b, 2a, 2b (see: 4.2.1), 2d, and 3d. Farnesoic acid carboxyl-O-methyltransferase (FAMT, PRG 1b) shows a similar amount of expression in control and inoculated plants, which is expected if its induction is reliant upon wounding or insects. A senescence associated and QQS (Qua Quine Starch) related protein (SAQR, PRG 2a) is known to increase in response to oxidative stress but it is also an indicator of senescence, and down-regulates right before cell death (Jones et al., 2016). The replications used for the RT-PCR could have had an overall accelerated disease progression compared to the tissue harvested for RNA-Seq, and thus there might be some discrepancy in the what constitutes the late stages of infection. A TPR (tetratricopeptide repeat) -like superfamily protein (PRG 2d) aids in osmotic stress tolerance but is also a hormone regulator (Schapire et al., 2006), and its consistent expression in both control and inoculated plants in the RT-PCR may indicate it is acting as a constitutive regulator. Sinapoylglucose malate sinapoyltransferase (SNG1, PRG 3d) displayed an increase on 28 DPI in the RT-PCR; by this stage in infection the shoots were mostly dead and increased cell turnover, a function of PRG 3d (Lehfeldt et al., 2000), could be a symptom of

accelerated cell death.

Disappointingly, further RT-qPCR analysis with more replicates for these 8 PRGs revealed no significant change ($p=0.05$) in expression between any DPI. This is likely due to the large standard error at each DPI time point. Consistent change in gene expression at any one DPI between different replicates was based on the assumption that infection starts at the same time for all inoculated plants. However, even in lab conditions this is difficult to verify. The initiation of infection is dependent on the germination of the spores, which is largely dependent on the maturity of the gall they were harvested from. Spores from mature galls were found to germinate earlier than those from younger galls (Macfarlane, 1970). Older clubroot-infected tissue is also more likely to have accumulated bacteria and fungi, which decompose host tissue and minimize contaminants when collecting spores (Ayers, 1944). The galls used for soil baiting were harvested from canola galls, around 35-40 days old. In one instance spores were collected from fresh gall, and other instances the spores were purified from dried gall kept at room temperature for an indeterminate amount of time. Regardless of spore concentration, which was consistent between replications, it is very likely that variation in the quality of these galls would have had an impact on spore germination, and therefor affect the stages of infection between replicates. This discrepancy is far more obvious in RT-qPCR results than in RT-PCR results, which is likely because the RT-qPCR results are an average of three replicates. However, due to the lack of consistent expression trends within the RT-qPCR data, it is not possible to confirm staggered infection times.

The RT-qPCR analyses were not in agreement with the RNA-Seq and RT-PCR data, as those displayed an identifiable difference in gene expression occurring between 17 and 20 DPI or 14 and 21 DPI, respectively. Additionally, a very minor phenotypic change can be seen as early as 12 DPI (Figure 3.5 A), which may be a dwarfing response (Cook & Schwartz, 1930). Large variations, though statistically insignificant, at 28 DPI in the RT-qPCR are representative of the advanced level of infection seen at 21 DPI in both shoots and roots (Figure 3.5 B, C). At 21 DPI there is still viable shoot tissue available for collection and extraction of RNA, however in multiple replicates by 28 DPI the housekeeping gene actin may have degraded beyond successful PCR amplification, thus making it an unreliable reference gene for gene expression in the PRGs. This phenomenon is observable in the RT-qPCR results as it includes three replicates. The RT-PCR results displayed a replication where disease progression allowed for the collection of viable 28 DPI tissue.

To accomplish my fourth objective, promoter-marker constructs were designed to include promoters of the individual PRGs and a modified existing fluorescent protein. Florescent protein markers were chosen based on their brightness, stability, and minimization of autofluorescence. As infection does not occur until well into the *Arabidopsis* life cycle, it is critical to have a bright reporter to ensure the intensity of fluorescence is still distinguishable in shoot tissue with large vacuoles. Photobleaching is also a concern as ideally we would like to observe changes in fluorescence over a period of days without compromising the reporter's effectiveness. Conversely, to generate accurate gene expression imaging, it is important that the fluorescent reporter not be considerably more stable than its co-expressed gene. This "over-stability" could indicate gene expression long after down-regulation of said gene. Mann et al. (2012) were able to show that tdTomato and mOrange, respectively a tandem dimeric and monomeric fluorescent protein, were among the brightest reporters in transgenic plant studies. During constitutive expression both proteins remained bright in older plants and exhibited minimal autofluorescence. Furthermore, localization of the proteins to the endoplasmic reticulum (ER) showed a noticeable increase in brightness. Prior to this, Shaner et al. (2008) constructed a highly photostable variant of mOrange, mOrange2, which maintains the favorable qualities of mOrange.

The tdTomato and mOrange2 sequences were modified to include an HDEL sequence, for FP retention in the ER. Constructs containing the Cauliflower Mosaic Virus (CaMV35S) and a tdTomato or mOrange2 sequence were also designed, unfortunately they did not clone in the initial stages of the project. Due to experimental difficulties in the cloning process and time constraints, only one final construct was achieved: the *GASA6* (PRG 3b) promoter with the mOrange2 reporter (*GASA6::mOrange2*). My final objective was achieved by transforming *Arabidopsis* with the *GASA6::mOrange2* construct and establishing transgenic lines ginger1 and ginger2.

Despite the inconclusive RT-qPCR results of the 8 PRGs selected for cloning, it is worth investigating the function and nature of these genes to determine if any direct relationship to disease progression can be made. Though for this project the function of the specific protein products was not particularly relevant in their selection as reporters, understanding these genes response to clubroot could increase our understanding of the events that occur in shoot tissue of clubroot-infected plants and may facilitate the selection of more appropriate PRGs in future studies.

4.2 Potential reporter gene functions

PRGs were categorized into four distinct groups based on RNA-Seq data for easier discussion of their expression patterns. This is also useful for making connections between gene function and expression. It is useful to note that primary PRGs that were not chosen for cloning also have gene functions that are associated with stress response or are involved in clubroot affected pathways.

Category 1 (Figure 3.1; 3.6) and 2 (Figure 3.2; 3.7) consisted of genes which were up-regulated in in response to clubroot. All Category 1 PRGs are related to plant defense mechanisms or stress responses. Anthocyanin 5-O-glucosyltransferase (*UGT75C1*, PRG 1c) is associated with resistance to biotic stressors (Gachon et al., 2005; Lorenc-Kukula et al., 2005), and chalcone synthase (*CHS,* PRG 1d) and dihydroflavonol 4-reductase (*DFRA,* PRG 1f) induce the accumulation of stress-associated flavonoids (Liang & He, 2018; Winkel-Shirley, 2002). Category 1 also contained the only gene with no known function, PRG 1a. Interestingly, though Category 1 PRGs were expected to up-regulate compared to a steady expression in the control, their expression remained steady and the discrepancy in expression levels was actually due to down-regulation in the control.

Category 3 (Figure 3.3; 3.8) and 4 (Figure 3.4; 3.9) consisted of genes which were down-regulated relatively lower and higher, respectively. Expansin A1 (*EXP1,* PRG 3a) and expansin A8 (*EXP8,* 3e) are responsible for cell wall expansion (Lee et al., 2001), and beta-galactosidase 1 (*BGAL1,* PRG 3f) encodes a cell wall protein known for cell wall remodeling (Moneo-Sánchez et al., 2016). Category 3 PRGs are involved with cell wall function, which undergoes a considerable amount of modification during infection. Plastid-specific ribosomal protein 1 (PRG 4b) stabilizes the ribosomal subunits and has been linked to chloroplast synthesis (Sharma et al., 2010). Its RT-PCR expression likely signals the general down-regulation of organelle biosynthesis in the later stages of disease progression.

Categories 1 and 3 were up- and down-regulated at lower levels than Categories 2 and 4. These groupings allowed for the comparison between levels of gene expression, as genes with higher levels of differential expression may prove to be better reporters.

4.2.1 Category 1 and 2 PRGs

Glutathione s-transferase 26 (*GST26*, PRG 1e) is part of the ubiquitously transcribed glutathione s-transferase (GST) family of multifunctional, dimeric enzymes that are involved in a large number of cell processes but are mostly known for their detoxification properties. The tripeptide glutathione covalently links to both endogenous and xenobiotic chemicals, such as anthocyanin and chemicals introduced to the plant through insect herbivory. The glutathione-conjugate is then sequestered from the cytosol to a vacuole (Coleman et al., 1997). Many secondary plant metabolites require localization into specific cell compartments to deter their natural phytotoxicity. It has long been established that anthocyanin is up-regulated in response to plant stress (Christie et al., 1994; Kovinich et al., 2015), however its presence in cytoplasm both prevents the production of more anthocyanin and is damaging to the cell. GSTs are involved in the pathway that tag anthocyanin related pigments for relocation to vacuoles (Marrs et al., 1995). There is also evidence that glutathione peroxidases can directly detoxify hydroperoxides, such as those that come from lipid peroxisomes (Bartling et al., 1993). Interestingly, *GST26* in wheat was induced by herbicides, but not *Puccinia recondita* or *Blumeria graminis* pathogens (Mauch & Dudler, 1993). This could suggest that *GST26* is specifically induced by the clubroot pathogen, which is ideal for a disease reporter.

The RNA-Seq (Figure 3.1 1e) and RT-PCR (Figure 3.6 1e) data both reflected this increase in expression in response to clubroot stress compared to controls. Notably the RT-PCR showed strong slightly up-regulated expression in inoculated shoots as early as post 7 DPI. There was also comparatively a higher difference in reads between 17 DPI control and inoculated shoots in the RNA-Seq, which coincided with the low level of expression in RT-PCR control plants in 14 DPI onwards. As plants age, ethylene induces plant senescence and auxin production is decreased (Burg, 1968). As many GSTs have been found to be auxin inducible (Droog et al.,1995; Takahashi et al., 1995), the down-regulation of *GST26* in control plants could be related to auxin down-regulation. RT-qPCR data (Figure 3.10) for *GST26* exhibits a similar trend as seen in RNA-Seq and RT-PCR expression 7 DPI onwards.

Lipid droplet associated protein 1 (*LDAP1*, PRG 2b) regulates the ER-derived lipid droplet biogenesis found in seeds (Pyc et al., 2017). Lipid droplet functions in leaves has not been widely researched, though there is evidence that they play a role in plant defense. Phytoalexins, synthesized

in the presence of pathogens (Glazebrook & Ausubel, 1994) were shown to be produced in leaf lipid droplets in *Arabidopsis* following infection with *Colletotrichum higginsianum* (Shimada et al., 2014). LDAP genes were also shown to be induced by ABA (Winter et al., 2007), a stress signal phytohormone. Drought stress, known to affect clubroot in its late infection stages (Cook & Schwartz, 1930) has been shown to induce *LDAP1* expression (Kim et al., 2016). However, *LDAP1* is up-regulated during both cold and heat stress whereas *LDAP3*, another main LDAP, was only up-regulated during cold stress (Gidda et al., 2016). This highlights the variability of LDAP function.

RNA-Seq (Figure 3.2 2b) showed high levels of expression of *LDAP1* by 24 DPI, which is consistent with the current research but incongruous with RT-PCR (Figure 3.7 2b) and RT-qPCR (Figure 3.11 2b) results. There was also consistently weak expression from 5 to 28 DPI in inoculated plants in the RT-PCR. RT-qPCR did not reflect any trend between DPI. It is possible that *LDAP1* has a unique response to clubroot infection, whereupon expression is down-regulated during initial infection from a disturbance in homeostasis and then up-regulated as disease progresses.

TPR (tetratricopeptide repeat)-like superfamily proteins (PRG 2c) are involved in a wide variety cellular functions, and act as protein interaction domains (Blatch & Lässle, 1999). Tetratricopeptide motifs are usually found in signaling pathways, such as those in phytohormones. These pathways can be indirect, such as *TPR thioredoxin-like 1*, which has no effect on ABA synthesis but regulates its function (Rosado et al., 2006). Phytohormone signaling pathways can also directly affect TPR regulation, such as the GA induced TPR SPINDLY, which in turn down-regulates of GA (Jacobsen et al., 1996).

RNA-Seq (Figure 3.2 2c) showed some expression of PRG 2c in the control plants, however this was not visible in the RT-PCR (Figure 3.7 2d). Furthermore, it was among the number of PRGs (1e, 2a, 2b, 3b, 3e, 4a) which displayed little to no expression in inoculated plants on 0 and/or 2 DPI, though this may be reflected in the actin expression. Due to the characteristic multifunctionality of the TPR-like proteins and no information known about this specific protein, there is not enough data to determine its function, however the RT-PCR showing steady expression in only the inoculated plant and its induction with GA suggests that this gene is stress inducible.

4.2.2 Category 3 and 4 PRGs

Gibberellin stimulated Arabidopsis 6 (*GASA6*, PRG 3b) is part of the GA-stimulated family of transcripts which are known to be GA-inducible and ABA-repressible (S. Zhang & Wang, 2008). This gene is regulated by the GA repressor protein GAI (GA Insensitive) and plays many roles in hormone crosstalk. TPR SPINDLY is also regulated by GAI, specifically it is epistatic to GAI (Jacobsen et al., 1996). As GA is a prominent phytohormone signaling component of stress pathways and GA stimulated growth is down-regulated during stress, down-regulation of GA inducible genes are anticipated to have lower expression in clubroot infected plants. This is apparent in both the RNA-Seq (Figure 3.3 3b) and RT-PCR data (Figure 3.8 3b). qRT-PCR (Figure 3.12 3b) data disagreed with the trends in the RNA-Seq and RT-PCR, as it appears to have no trend other than a slight up-regulation on 21 DPI.

Endoxyloglucan transferase (*EXGT-A1*, PRG 3c) is a glycoprotein enzyme which can both split xyloglucan molecules and also link xyloglucan molecules to other xyloglucan molecules (Nishitani & Tominaga, 1992). Xyloglucans crosslink cellulose microfibrils in the cell wall matrix, and their division and reattachment allow for cell expansion. In shoot tissue during clubroot infection, there was a down-regulation of this gene during later stages of infection (Figure 3.3 3c; Figure 3.8 3c). Up-regulation in roots is IAA induced and allows for cell wall expansion (Devos et al., 2005) to cause gall formation. By 21 DPI, the gall has already become a major resource and hormone sink, resulting in the down-regulation of *EXGT-A1* and small cell size in the shoot tissue. Closely related to EXGT-A1 function, expansin A8 (*EXP8,* PRG 3e) belongs to the alpha-expansin family and is also involved in auxin-induced cell expansion. Whereas EXGT-A1 links cellulose, EXP8 links cellulose to hemicellulose (Cosgrove, 2000).

EXP8 also follows the same expression pattern (Figure 3.3 3e; Figure 3.8 3e) as *EXGT-A1*. They work in conjunction to loosen the cell wall for gall formation in roots, and impede cell growth during nutrient and drought stress in shoots (Devos et al., 2005). RT-qPCR (Figure 3.12 3e) data trends show a slight decrease in expansin expression in inoculated plants from DPI 0 to DPI 14 before a slight up-regulation on DPI 21. As gall formation is still underway at this stage of infection, it is possible that the root acts as a source for excess auxin.

The aluminum induced protein with YGL (Tyr-Gly-Leu) and LRDR (Leu-Arg-Asp-Arg) motifs (*AILP1*, PRG 3g) has been associated with inducing cell elongation (Lally et al., 2001) and pathogen resistance (Hamel et al., 1998). TAl-18 (Triticum aluminum 18), a protein synthesized during Al toxicity in wheat, was found to show a high degree of homology with a pathogen-response gene in celery (Cruz-Ortega & Ownby, 1993) suggesting these two stressors cause common stress responses. RNA-Seq and RT-PCR (Figure 3.3 3g; 3.8 3g) both display a very slight decrease in expression in inoculated plants. Based on literature closely relating the down-regulation of cell wall genes to clubroot infection, the inhibition of cell elongation could be the cause of *AILP1* down-regulation, through an Al mediated pathway.

As plant cells are not fully separated due to sharing their plasma membrane through plasmodesmata, cell wall-plasma membrane linker proteins (*CWLP*, PRG 4a) are needed to maintain communication between the plasma membranes and the cell wall. This is especially important as the plasma membrane contains a multitude of polysaccharides that perform signaling roles, including those related to pathogenesis (Vorwerk et al., 2004). One category of these proteins is proline-rich proteins (PRP). The presence of proline in the cytosol and chloroplasts was found to induce CWLP synthesis (Stein et al., 2011). Generally, free proline accumulation occurs during osmotic and microorganism stress (Fabro et al., 2004). However, RNA-Seq and RT-PCR (Figure 3.4 4a; 3.9 4a) show a down-regulation of expression, so it is likely this PRG is a different variant of CWLP with a different function. The up-regulation trend seen in the RT-qPCR (Figure 3.13) could be too minimal to see in RT-PCR gels.

In general, genes involved in plant stress signaling or defense were up-regulated or maintained expression in shoots during clubroot infection, which could be anticipated. Genes involved in cell wall or plasma membrane modification were down-regulated, which coincides with current literature. Negatively regulated hormones auxin and GA were down-regulated through different signaling pathways and altered expression in other genes in their respective signaling pathways, including *GASA6* and *EXGT-A1*.

4.2.3 Root-shoot dynamics

As clubroot infection begins in the roots, it is important to relate gene expression in the shoot to what is being expressed in the root. Signs of clubroot infection were visible in the roots as early as 16 DPI, with obvious gall formation by 20 DPI (Irani et al., 2018). It is within this time frame that infected cells within the root are producing auxin to increase cell size (Ludwig-Müller, 1999) and promoting cell division through cytokinin biosynthesis (Muller & Hilgenberg, 1986). This drastic change in root physiology is reflected in the differential gene expression seen in the shoots during these DPI. Perhaps surprisingly, it was found that fewer genes are differentially expressed in the root on 17, 20, and 24 DPI compared to those in shoots (Irani et al., 2018). Genes up-regulated in shoots and roots included those related to glutathione-mediated detoxification and quercetin glycoside biosynthesis. The up-regulation of *GST26* in shoots corresponds to its expression in the roots. It is possible this gene is playing the same detoxification role in both tissues. Flavonoid pathways induced by GSTs result in the increase of quercetin glycoside, which have been found to reduce beta-galactosidase in apples, a response that can also be seen in the shoots of *Arabidopsis* (Dick et al., 1985). Genes down-regulated in shoot and roots included those related to homogalacturonan degradation and phosphate acquisition. Unsurprisingly, homogalacturonan is a pectic polysaccharide which is a component of the primary cell wall, and involved in cell wall expansion (Wolf et al., 2009). This is comparable to the down-regulation of CWLP also seen in shoots. Nutrient acquisition becomes overall limited as the gall continues to develop and act as a nutrient sink, which can be seen in the shoots as drought stress responses are activated. In contrast, cell wall modification genes are up-regulated in the roots and down-regulated in the shoots. This accommodates gall development in the roots and the beginning of cell degradation in the shoots. Evaluating changes in all host tissues provides a more inclusive understanding of pathogen-host interactions as a whole.

4.3 Future considerations

A non-destructive fluorescent-based method of detecting the early stages of clubroot infection would be an asset in further clubroot research, and I believe it is worthwhile to pursue this concept. Though knowledge of the gene function is not necessarily important to identify a suitable disease reporter, continued investigation of their functions and responses to different stressors could

be useful to determine if certain gene families are more reliable reporters. Differential expression of genes that are not seen in other biotic or abiotic stress responses could indicate that the reporter is highly disease specific. The gene candidates chosen for this project were found to be responsive to many types of abiotic or biotic stress, or had relatively unknown specific functions, which makes it unlikely that any candidates would be disease specific.

Moving forward I would suggest the following improvements, in order of predicted efficacy. The current time-course may be more appropriate for *Plasmodiophora*, where pathogen gene expression occurs in the first few days of infection, than *Arabidopsis* shoots, where infection symptoms are present last. Specifically, the RNA-Seq data show changes in gene expression occurring between DPI 17 and DPI 20, and my RT-PCR results show changes in gene expression between DPI 14 and DPI 21—these time points neglect differential gene expression in two crucial time intervals. The first is between DPI 14 and DPI 17; wherein significant differential expression of a gene during this stage of infection would be the ideal reporter gene for early detection, as we know DPI 17 is pre-infection and can generally see there is a qualitative difference in expression between DPI 14 and DPI 21. The second is between DPI 17 and DPI 20; the RNA-Seq confirms that there is significant differential gene expression during this interval, however we can only infer levels of up- or down-regulation between these time points. Focus should instead be shifted to 14, 15, 16, 17, 18, 19, and 20 DPI. RNA-Seq, RT-PCR, and RT-qPCR all suggest this time interval is when the most drastic changes in gene expression occur.

Differing infection times and disease progression rates were potential causes for high standard error within DPI. To minimize the variation in disease progression between replicates, multiple replicates should be inoculated with the same spore solution and the time-course run simultaneously. Finally, parameters applied to the RNA-Seq data for the initial selection of PRGs could be relaxed. This would allow for genes with more variation between control and inoculated plants, which may prove to show significant difference between DPI in the subsequent modified time-course. Additionally, the RNA-Seq data could be utilized for differential co-expression analysis, which would further elucidate which genes are being differentially expressed during infection.

4.4 Conclusion

Some PRGs displayed favourable trends initially, however ultimately were not viable candidates for early detection of clubroot infection. The difficulty of verifying consistent disease progression with soil baiting may have slightly distorted the results of the time-course. Nevertheless, the results were compared to differential gene responses from other biotic and abiotic stresses, which was important for understanding the role of the PRG within clubroot infection. Several expression patterns in my chosen PRGs are reflected in wider *Plasmodiophora* host transcriptome studies. A common trend among genes I selected and those in transcriptome studies was the down-regulation of cell wall biosynthesis genes (Jubault et al., 2013), specifically a decrease in expansins in shoots (Irani et al., 2018). This trend further highlights the large role cell wall modification plays in clubroot disease. Selected cell wall-unrelated genes were generally up-regulated plant defense genes, which is has further been identified in the previously mentioned studies. As a consequence of working with few genes compared to RNA-Seq, the expression of my selected genes reflected the trends seen in genes that fall within the same functional category in the literature. No novel clubroot specific reporter gene was identified, but further investigation into generating research tools will aid in making clubroot easier to work with in a lab setting, and also allow for more research opportunities into clubroot management.

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